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14. ABSTRACT
Our data demonstrates that WBP-2 is recruited onto the hormone responsive promoters in the presence of hormone and it specifically enhances the transactivation functions of PR and ER. Our data also demonstrates that WBP-2 contains an intrinsic activation domain and the cPPXY of WBP-2 is essential for its coactivation and intrinsic activation functions. Our preliminary data also demonstrates that the WBP-2 binding protein, YAP1 enhances PR and ER transactivation but YAP1's coactivation function is absolutely dependent on WBP-2. Furthermore, cPPXY motif of WBP-2 and WW-domain of YAP1 is required for YAP1 to work as a transcriptional coactiva-tor. Additionally, our data also indicate that the coactivation functions of WBP-2 and YAP1 are suppressed by WWOX1, suggesting that WWOX1 may regulates the transactivation functions of ER and PR by antagonizing the functions of WBP-2 and YAP1. Taken together our data estab-lished the role of WBP-2 and YAP1 as coactivators and WWOX1 as a repressor for ER and PR transactivation pathways.

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Introduction

Estrogen (ER) and Progesterone (PR) Receptors*:* Estrogen receptor-α (ER) and progesterone receptor (PR) are members of a superfamily of hormone-regulated transcription factors that stimulate gene expression in response to estrogens and progesterones respectively. These receptors contain common structural motifs, which include a less well-conserved amino-terminal activation function (AF-1) that affects transcription efficiency, a central DNA-binding domain, which mediates receptor binding to specific DNA enhancer sequences and determines target gene specificity, and a carboxy-terminal hormone-binding domain (HBD). The HBD contains activation function-2 (AF-2), the region that mediates the hormone-dependent activation function of receptors. In order to activate gene transcription, the ER and PR undergo a series of well-defined steps. When bound to hormone, these receptor undergo a conformational change, dissociation from cellular chaperones, receptor dimerization, phosphorylation, interaction with co-activators and recruitment of chromatin modifying enzyme activities such as histone acetyl transferase activity (HAT), methyl transferase activity, ATPase activity, ubiquitin-conjugation activity and ubiquitin-protein ligase activity, DNA-binding at an enhancer element of the target gene, and subsequent recruitment of basal transcription factors to form a stable preinitiation complex [37-40]. These events are followed by up- or down-regulation of target gene expression (Fig. 1). However, these receptors may also be converted into active forms even in the absence of hormones in target cells. The mechanism of hormone-independent activation of ER and PR has not been understood fully yet but it may involve the bypassing of any one of the above mentioned steps of hormone-dependent activation [41, 42].

Coactivators of ER and PR: Nuclear hormone receptor coactivators are molecules that interact with activated receptors and stimulate receptor-mediated transcription of target genes. There are now ~200 published nuclear hormone receptor coactivators that work with ~ 48 nuclear receptors [6, 8, 9, 11]. The most widely studied coactivators include members of the p160 family of coactivators; SRC-1 (steroid receptor coactivator-1) [43], SRC-2 (TIF-2; transcription intermediary factor-2/GRIP-1; glucocorticoid receptor interacting protein-1) [44, 45], SRC-3 (p/CIP; p300/CBP interacting protein/ACTR; activator of thyroid and retinoid acid receptors/AIB-1; amplified in breast cancer-1/RAC-3; retinoid acid receptor coactivator-3/TRAM-1; thyroid receptor activator molecule-1) [46-49], the CBP (CREB-binding protein)/p300 family [50], coactivator-associated arginine methyltransferase (CARM-1) [51, 52], and E6-AP [10]. We have previously reported the cloning and characterization of E6-AP as a novel dual function steroid hormone receptor coactivator. Additionally, we also demonstrated that the E2 ubiquitin-conjugating enzyme, UbcH7, acts as a coactivator of steroid hormone receptors [53]. Initially, it was thought that coactivators act as adaptors and provide a bridge between DNA binding transcription factors and the general transcription machinery. This simple scenario of coactivator action turned out to be much more complex. It has been shown that coactivators can mediate chromatin modifications either through acetylating reactions mediated by histone acetyl transferases or through nucleosome remodeling complexes [6, 8, 11]. Coactivators are predicted to have many activities in addition to the initiation of transcription, such as mRNA transport from the nucleus, mRNA translation, and posttranslational modifications of the synthesized protein. That coactivators possess stratified actions in the entire process of transcription-translation reflects the fact that they do not act alone but rather as part of multiprotein complexes. These multisubunit entities, containing many individual enzymatic activities, represent a complex machine that is able

to concentrate and link diverse enzymes, and the processes that they regulate, together in one place [6, 8, 11]. In this way, the coactivator complex executes the coactivator's final agenda—that is, to see a particular gene expressed as a mature functional protein.

Transcription is a highly dynamic and orderly process involving many subreactions (multiple steps of initiation, elongation, splicing, and termination) [54]. Given that so many nuclear hormone receptor coactivators have been identified, there is certainly no shortage of them to participate in the wide variety of transcription subreactions. But why would a cell possess such a cumbersome transcriptional apparatus? The answer may lie in the fact that mammals are substantially more complex than organisms such as yeast, worm, and the fruit fly, which have far fewer nuclear receptor coactivators. For instance, only a single nuclear receptor coactivator (Taiman/dAIB1) has been identified in fruit flies so far [55].

For transcription to proceed, there need to be histone modifications (such as acetylation and methylation), ATPase-dependent chromatin remodeling, initiation of transcription, elongation, alternative RNA splicing and mRNA processing, and termination. The focus of coactivator enzymatic activities in these processes has centered on the posttranslational modification of histones and chromatin [56, 57]. However, it is becoming clear that nuclear hormone receptors and their coactivators are also subject to posttranslational modification [58, 59]. The posttranslational targeting of nuclear hormone receptors and their coactivators is important because these modifications influence the expression of functionally related groups of genes. *Identification of new coactivators will provide the prime source for the discovery of new molecular events in transcriptional reactions.*

As mentioned above that coactivator proteins form multiprotein complexes to efficiently regulate target gene transcription. Recently, we have identified novel protein named WBP-2 as an E6-AP interacting protein and show that it specifically modulates ER and PR functions. WBP-2 was previously shown to interact with human YAP1 via the WW-domain of YAP1 protein [12]. The WW-domain is characterized by 35-40 semi-conserved amino acids, which are involved in protein-protein interaction. WBP-2 interacts with the WW-domain via a short proline-rich motif (PPXY) with the consensus sequence of four consecutive prolines followed by tyrosine [16, 31, 35]. It has been speculated that WBP-2 plays role in transcription, but its exact function in steroid hormone receptor-dependent transcription has not been defined yet. Additionally, it has been suggested that YAP1 may also regulate transcription by acting as a coactivator for several transcription factors but what role YAP1 plays in steroid hormone receptor-mediated gene transcription remains unknown. *The main goal of this proposal is to decipher the mechanism by which WBP-2 and YAP1 regulates ER and PR-mediated transcription.*

WBP-2 as a Coactivator: WBP-2 is a 30 kDa protein that contains three proline-rich motifs known as PPXY motifs. WBP-2 was originally identified as a putative ligand that binds to the WW-domain of YAP1 with relatively high affinity and specificity. The PPXY motifs of WBP-2 are distinct from the PXXP ligand consensus sequence of Src homology domain 3 (SH3) domains [16, 31, 35]. The exact function of the WBP-2 and its PPXY motifs remain unknown. Recently, we cloned WBP-2 as E6-AP interacting protein. Despite the fact that E6-AP does not contain a WW-domain, our data clearly demonstrate that WBP-2 can also interact with proteins that do not contain a WW-domain suggesting a new type of protein-protein interaction between PPXY motif containing protein and *HECT* (homologous to the E6-AP carboxy terminus) domain con-

taining proteins [60, 61]. Additionally, we also found that WBP-2 specifically directly interacts with ER and PR. Our observation that WBP-2 can interact with proteins that do not contain WW-domain is also consistent with a previously published study that suggests WBP-2 interacts with the transcription factor Pax8 [14]. Previously, it has been suggested that WBP-2 act as a transcriptional adaptor for Pax8 [14]. We have demonstrated a role for WBP-2 in steroid hormone receptor functions. We have shown that WBP-2 specifically enhances the hormone-dependent transcriptional activity of ER and PR suggesting that WBP-2 can act as transcriptional coactivator of selected steroid hormone receptors. Furthermore, we have also demonstrated that the most carboxy terminal PPXY motif (cPPXY) of WBP-2 is required for its coactivation function, suggesting a role for the PPXY motif in transcription. Together, our results indicate the role of WBP-2 as a coactivator in modulating ER and PR functions [12]. Previously, it has been postulated that the PPXY motif plays an important role in transcriptional activation since the PPXY motif is present in the transcriptional activation domains of a wide range of transcription factors including c-Jun [62], AP-2 [63], NF-E2 [22], C/EBP〈 [64] and PEBP2/CBF [30]. Our data indicate a role for the PPXY motif and WBP-2 in ER and PR transactivation, but the exact mechanism by which WBP-2 acts as a coactivator and the role of the PPXY motif in ER and PR-dependent gene activation is not fully understood. Furthermore, *the actual target proteins of the PPXY motif that confer transcription stimulation activity have not yet been identified.* Therefore, there is a clear need *to understand the role of WBP-2 and its PPXY motif in ER and PR-mediated gene activation and transcription.*

WW-Domain Containing Proteins as Modulators of ER and PR Function: Specific protein-protein interactions and multiprotein complexes are important for a multitude of cellular processes including gene transcription. As mentioned above that WBP-2 binds to proteins that contain WW-domain. WW-domains are small protein modules composed of 38-40 amino acids and fold as a stable, triple stranded beta-sheet. The name refers to two signature tryptophan (W) residues that are spaced 20-22 amino acids apart and are present in most of the WW-domains. In some instances, however, the first or the second conserved tryptophan is substituted by other aromatic residues. WW-domains bind to their ligands via the proline rich peptide motifs. WW-domains can be grouped into four classes according to their ligand binding preference. Class I includes WW-domains that bind to the PPXY core sequence which is present in WBP-2. The example of Group I WW-domain containing protein is YAP1. Class II WW-domains prefer ligands containing a stretch of prolines interrupted by a leucine. Class III includes WW-domains interacting with proline-rich sequence that contains arginines or lysines. WW-domains binding phosphoserine or phosphothreonine followed by a proline residue are grouped in class IV. WW-domains are found in both cytoplasmic and nuclear proteins, WW-domains containing proteins are involved in a wide variety of cellular processes, including ubiquitination, nuclear signaling, cell cycle control, transcriptional regulation and the recruitment of signaling proteins [28, 31, 35]. Since, WW-domains bind to the PPXY motif and WBP-2 has been shown to contain PPXY motifs, an important step toward characterizing coactivation function of WBP-2 is to identify to which particular WW-domains it bind, and hence determine the mechanism by which it act as a coactivator and also find out with which signaling pathway it is involved. In order to identify the WW-domains that could interact with the WBP-2 protein, we utilized Panomics' TranSignal WW-domain Array (Panomics Inc., CA, USA). This array contains 67 different human WW-domains from 42 different proteins. Our screening data suggest that WBP-2 interacts with a wide variety

of WW-domain containing proteins (please see preliminary data section) including YAP1, a transcriptional coactivator and WWOX1, a tumor suppressor.

YAP1 was originally identified as a protein binding to the SH3 domain of the Yes proto-oncogene product that belongs to the *src* family of protein-tyrosine kinases. YAP1 is a 65 kDa protein with a well characterized WW-domain [13]. Recently, a splicing variant that encodes human YAP with two WW-domains has been identified. YAP1 with a single WW-domain, known previously as human YAP, is renamed as YAP1 and the new YAP with two WW-domains is named as YAP2. The difference in the amino acid sequence between YAP1 and YAP2 is an insertion of the additional WW-domain in YAP2. YAP1 and 2 contain activation domain which is similar to VP-16 activation domain. Roles of YAP1 and 2 in transcription are not well defined [21]. Recently, YAP1 and YAP2 have been shown to be transcriptional coactivators for several genes but their transcriptional coactivation functions are dependent on PPXY motif containing proteins as in the case of ErbB4 (PPXY motif containing protein) signaling [21]. YAP1 and YAP2 interact with ErbB4 and are recruited to its specific target gene promoter. It has been suggested that YAP2 is a stronger coactivator of transcription than YAP1. Furthermore, phosphorylation of YAP1 and YAP2 by specific kinases regulates their cellular distribution and transcriptional coactivation functions. It is shown that Akt-dependent phosphorylation of YAP1 and YAP2 at Serine 127 induces the interaction of YAP1 with 14-3-3 and promote YAP1 and YAP2 localization to the cytoplasm resulting in loss from the nucleus where they functions as coactivators of transcription [65]. Our preliminary data also indicate a role for YAP1 in steroid hormone receptor-mediated transactivation function. We show that YAP1 expression itself has no significant effect on ER and PR-mediated transcription [12], however, when YAP1 was coexpressed with WBP-2, it could selectively modulate ER and PR-dependent gene transcription. Furthermore, mutation (W199F) in the WW-domain of YAP1 abolishes its coactivation functions [21]. The coactivation function of YAP1 is strictly dependent on the PPXY motif of WBP-2. *The mode of action of WBP-2:YAP1 complex in ER and PR-mediated transactivation is not yet understood.* In this proposal, *we intend to dissect the molecular mechanism and significance of this interaction with relation to ER and PR function.*

As mentioned above, in addition to YAP1, we have also identified another WW-domain containing protein, WWOX1 (a tumor suppressor) as a WBP-2 interacting protein in our WW-domain array. WWOX1 was originally cloned as a putative tumor suppressor gene that spans one of the most common active fragile sites in the human genome, FRA16D. WWOX1 is located on chromosome 16q23.3 and exhibits genomic alterations in several cancer types and a recent study showed a possible involvement of methylation in the regulation of WWOX1 expression. WWOX1 expression level is high in normal tissues; its expression is highest in hormonally regulated normal tissues such as testis, prostate and ovary suggesting that WWOX1 may play an important role in hormone regulated cancers [32, 33, 66-71]. This suggestion is supported by the recent publications which show that WWOX1 expression is frequently altered in tumor tissues and introduction of WWOX1 into WWOX1-negative breast and prostate tumor cells resulted in tumor suppression and apoptosis both *in vitro* and *in vivo* [72]. The WWOX1 contains two WW-domains. The tandem WW-domains of WWOX1 play an important role in WWOX1 function. Both WW-domains of WWOX1 contain a central core of two consecutive aromatic amino acids and therefore belong to the class I specificity of domains, which recognize ligands with the PPXY consensus motif. Recent mapping of the WW-domain in the human proteome identified a

repertoire of PPXY-containing ligands that bind to individual domains of WWOX1. The first WW-domain of WWOX1 bound 18 and the second WW-domain bound 16 ligands, all with PPXY consensus. The mapping data clearly documented that although the second WW-domain of WWOX1 contains a tyrosine in the place of the second conserved tryptophan, the signature residue directly involved in ligand binding, the specificity of the "WY domain" toward PPXY core motif was not changed [33]. Previously, it has been shown that WWOX1 interacts with PPXY motif containing proteins, p73 and AP-2 and suppresses their transcriptional activities [36, 66, 73, 74]. Most recently, it has been shown that WWOX1 interacts with another PPXY motif containing protein, ErB-4 and compete with YAP1 for ErB-4 binding and suppress the coactivation functions of YAP1 [36]. Since, WWOX1 and YAP1 have the similar tandem WW-domains and both interact with common protein, WBP-2 but have different effects on transactivation, in this proposal, *we will examine whether WWOX1 and YAP1 expression have opposite effects on ER and PR-dependent gene transcription.*

PPXY Motif and WW-Domain; Possible Function in Gene Regulation: Protein-protein interaction modules are important for the proper signal transduction process in any cell. There are numerous such modules that mediate various processes from cell cycle progression to arrest and eventually apoptosis. One such module, the PPXY motif and WW-domain (PPXY-WW module) has gained prominence in the last decade. WW-domains mediate their interactions with proteins that contain a short PPXY motif. PPXY motif containing proteins essentially act as ligands for WW-domain containing proteins. The K_d of interaction for PPXY motif and WW-domain complex formation is in the high nM to low ⌠M values. Phosphorylation of the terminal tyrosine in the PPXY motif and phosphorylation of WW-domains containing proteins by specific kinases abolishes their interaction, suggesting that this modification represent a negative regulation mechanism for PPXY-WW module. Although the precise physiological roles of the PPXY motif and WW-domain remain undetermined, their presence in diverse proteins involved in signaling, regulatory, transcription and cytoskeletal functions, as well as their rapidly emerging role in signaling mechanisms that underlie several human diseases, clearly underscores their importance. Protein-protein interactions involving WW-domains and PPXY motif have been implicated in many diseases, including muscular dystrophy, Liddle's syndrome, Alzheimer's, Huntington disease and Cancers [16, 31, 35, 75].

Many new roles of the PPXY motif and WW-domain in the nucleus and transcription are just emerging. It is interesting to note that the PPXY motif is found in the transcriptional activation domains of many transcription factors and mutations in the PPXY motif either reduce or abolish their transcriptional activities. This observation suggests that the PPXY motif plays a role in mediating transcription stimulation by interacting with WW-domain containing proteins which act as transcriptional coactivator. Since, the interaction between WW-domain and PPXY motif is highly specific, it suggests that PPXY-WW complex is a more specialized coactivator complex of a subset of transcription factors [16, 21, 31, 35, 75]. This suggestion was supported by our observation which shows that PPXY motif containing protein, WBP-2 and WW-domain containing protein, YAP1 specifically coactivate ER and PR-dependent transcription where as this protein complex has no significant effect on the transactivation functions of other receptors. In contrast to this complex, it has been shown that other transcriptional coactivator complexes such as CBP, SRC-1 and p/CAF (p300/CBP associated factor) coactivates the transcriptional activities of a variety of receptors without exerting any specificity [6]. In addition to transcription stimu-

lation, the PPXY motif has also been shown to suppress transcription after interacting with certain WW-domain containing proteins. These observations suggest that *PPXY-WW module is involved in gene transcription but the actual mechanism by which PPXY-WW complex modulates transcription remains unknown.*

Significance: Coactivators play important roles in diverse pathological processes, such as cancer, inherited genetic diseases, metabolic disorders, and inflammation [6]. There is little doubt that we have much to learn about the biologically diverse roles of coactivators and that we have only scratched the surface of this expansive coactivator cosmos. Therefore, characterizing the mechanism of action of coactivator proteins will provide the prime source for the discovery of new molecular events in transcriptional reactions *and their role in cellular, physiological and pathological processes.* Collectively, our preliminary data suggests that WBP-2 (PPXY motif), YAP1 (WW-domain) and WWOX1 (WW-domain) are key regulators of ER and PR transactivation function but the precise roles of the PPXY motif and WW-domain containing proteins in steroid hormone receptor signaling [12], cell growth and carcinogenesis remain undetermined. Protein-protein interactions involving WW-domains and PPXY motif have been implicated in many diseases, including hormone regulated cancers. But the exact mechanism by which they regulate transcription, cell function and growth are largely unknown. Novel concepts and approaches to elucidate the molecular mechanisms by which PPXY motif and WW-domain containing proteins regulates hormone-dependent gene transcription are proposed here. Thus, accomplishing the specific aims outlined in this proposal will address the novel roles of the PPXY-WW complex in ER and PR function and will provide new and timely insights into the mechanism of action of PPXY motif and WW-domains in ER and PR signaling and cellular pathways that are regulated by these modulatory proteins.

Body

In the original proposal, we proposed to dissect the role od WBP-2 in ER and PR signaling and breast tumorigenesis.

- **Role of PY motif containing protein, WBP-2 in ER and PR signaling**
- **Role of WW-domain containing protein, YAP in SHR function**
- **Expression analysis of endogenous WBP-2 protein**

A. Role of PY motif containing protein, WBP-2 in ER and PR signaling

The second aim of the project is also the most elaborate so we started to concentrate in accomplishing this aim first. Though the original proposal contained a lot of preliminary data suggesting that WBP-2 is a coactivator of ER and PR, we methodically established that WBP-2 is indeed a coactivator of ER and PR.

I. Establishing WBP-2 as a coactivator of ER and PR

Isolation of WBP-2*:* In target cells, coactivators exist as multiprotein complexes, in order to identify the proteins that interact with E6-AP; we used yeast two-hybrid screening system. In this screen, we identified and cloned WBP-2 as an E6-AP interacting protein. We further show that WBP-2 specifically regulates ER and PR signaling [12].

WBP-2 Interacts with Estrogen and Progesterone Receptors*:* WBP-2 was cloned as E6-AP interacting protein and E6-AP has been shown to act as a coactivator for certain nuclear and steroid hormone receptors. To determine whether WBP-2 also interacts with steroid hormone receptors, we assayed WBP-2 binding to ER and PR in GST-pulldown assays. Fig. 2A shows strong WBP-2 interaction with full-length ER in the presence of estradiol (E_2) and reduced binding in the absence of hormone (-H) and presence of anti-estrogen, tamoxifen (T). Under similar conditions, WBP-2 was unable to interact with control GST protein. Similarly, WBP-2 and PR interaction was observed in the presence of progesterone, while only minimal binding was observed in the absence of hormone and presence of anti-progestin (RU486) (data not shown). In contrast, no interaction was observed between WBP-2 and other steroid receptors such as androgen receptor (AR) and glucocorticoid receptor (GR) (data not shown) [12]. These findings indicate that *WBP-2 specifically interact with ER and PR in the presence of hormone in vitro.* This result is consistent with previously published reports indicating that coactivators interact with receptors primarily only in the presence of hormone. It has been suggested that most coactivators interact with receptors via the LXXLL motifs contained within the coactivators. *WBP-2 is distinct from these coactivators, since, it does not contain LXXLL motifs and it specifically interacts with certain steroid receptors.*

WBP-2 and E6-AP Coprecipitate with ER in Cellular Lysates: Though WBP-2 could bind to both E6-AP and ER *in vitro,* we wanted to determine if all three proteins are present in a complex *in vivo* in the presence of hormone. Immunoprecipitation assays using MCF-7 cell lysates treated with no hormone (-H), estradiol (E_2) or anti-estrogen, tamoxifen (T) showed that both WBP-2 and E6-AP strongly interact with ER in the presence of estradiol (E_2) (Fig. 2B). However, a weaker interaction of E6-AP, WBP-2 and ER was also observed in the absence of hormone (-H) and tamoxifen (T). This observation is in agreement with our *in vitro* assays and furthermore, it suggests that *WBP-2, E6-AP and ER form a complex in vivo.*

WBP-2 Selectively Modulates the Transcriptional Activities of Progesterone and Estrogen Receptors*:* Since E6-AP acts as a coactivator for nuclear hormone receptors and WBP-2 physically associates with E6-AP and steroid hormone receptors such as ER and PR, we next tested whether WBP-2 regulates receptor-dependent activation of target gene

expression using transient transfection assays in T47D and MCF-7 cells. These assays were carried out with or without coexpression of WBP-2. WBP-2 had little effect on the transactivation functions of PR (T47D cells) and ER (MCF-7 cells) in the absence of hormone and anti-hormone. However, WBP-2 significantly enhanced the hormone-dependent transcriptional activities of PR and ER in T47D and MCF-7 cells respectively (Fig. 3A). In contrast, WBP-2 had only minimal effect on the transcriptional activities of GR and AR both with and without their cognate ligands (Fig. 3B). Similarly, WBP-2 had only negligible effect on other nuclear receptors like retinoic acid receptor-alpha, thyroid receptor and other transcription factors like p53 and VP-16 (data not shown) [12]. It has been reported that most of the cloned coactivators exhibit little receptor specificity and are able to coactivate a wide variety of nuclear hormone receptors. Unlike these coactivators, *WBP-2 exhibits receptor selectivity and preferentially coactivates the hormone-dependent transcriptional activities of PR and ER, and having little effect on the transactivation functions of other receptors.* ***These data suggest that WBP-2 selectively modulates the ligand-dependent transcriptional activities of ER and PR.***

Depletion of Endogenous WBP-2 Levels Reduces the Transcriptional Activity of PR and ER: To confirm that WBP-2 is indeed required for PR activation, cellular WBP-2 expression was down regulated in HeLa cells using WBP-2-directed siRNA. HeLa cells were transiently transfected with siRNAs directed against either WBP-2 or against GAPDH control, together with PR and PR-responsive reporter plasmids. Expression of siRNA directed against WBP-2 reduced the level of WBP-2 expression, while the control siRNA had no effect on WBP-2 expression (Fig. 4A). ***Depletion of endogenous WBP-2 reduced PR transcriptional activity by 75.3% (please refer attached manuscript), indicating that WBP-2 is required for fully functional PR activity.*** We further investigated whether disruption of WBP-2 expression also affects estrogen-dependent biological actions in ER-positive MCF-7 breast cancer cells. MCF-7 cells were transfected with two control siRNAs (siScrambled or siGAPDH) or siWBP-2, then cells were treated with either vehicle or estradiol and expression of endogenous estrogen-regulated gene, pS2 was measured by quantitative real time-polymerase chain reaction (RT-PCR). Upon treatment with estradiol, pS2 mRNA was significantly induced in cells that were transfected with either of the control siRNAs (siScrambled or siGAPDH) (Fig. 4B). Importantly, cells treated with siWBP-2 had a reduced *pS2* expression, *indicating WBP-2 is required for biological activity of ER in MCF-7 cells (Fig. 4B).*

Recruitment of WBP-2 Onto an Estrogen-Responsive Promoter in MCF-7 Cells: To further substantiate the coactivation function of WBP-2, we employed chromatin immunoprecipitaion (ChIP) analysis to assay the recruitment of WBP-2 onto an ER-responsive promoter in MCF-7 cells. Formaldehyde crosslinked protein-chromatin complexes were immunoprecipitated from MCF-7 cells that were treated with or without estrogen using the appropriate specific antibodies. The precipitated genomic DNA associated with ER or WBP-2 was amplified by polymerase chain reaction (PCR) using primers specific for estrogen receptor binding site within the pS2 promoter. ChIP analyses demonstrated the recruitment of WBP-2 onto ER-responsive promoter in the presence of estrogen (Fig. 5A&B) thus as is the case for known ER coactivators such as E6-AP, UbcH7 and SRC family members, WBP-2 is also recruited to target promoter in a hormone-dependent manner. This assay demonstrates

that *WBP-2 is physically present on an ER target gene promoter at a time when the gene is activated.*

***The Carboxy-Terminal PPXY Motif (cPPXY) of WBP-2 is Also Required for Its Coactivation Function**:* WBP-2 contains three proline-rich motifs containing the consensus sequence PPXY. The PPXY motifs are present in the transcriptional activation domains of several transcription factors, including c-Jun, AP-2, C/EBPα, NF-E2, KROX-20, MEF2B and PEBP2 suggesting that the PPXY motifs may play vital role in gene transcription [22, 30, 62-64, 76, 77]. PPXY motifs in these proteins have been previously shown to mediate protein-protein interactions and they represent potential transactivation domains that could function by recruiting additional strong transactivators to the promoters of target genes. The PPXY motifs in WBP-2 are binding recognition sites for a subclass of WW-domains present in certain other proteins. Since WBP-2 specifically enhances the transcriptional activities of ER and PR, we wished to determine whether the PPXY motifs of WBP-2 are essential for its ability to modulate the transcriptional activity of these receptors. To this end, we mutated each of the three PPXY motifs of WBP-2 to AAXA (A: alanine) (Fig. 6A) and tested the ability of the mutant WBP-2 proteins to modulate PR transcriptional activity by transient transfection assays. As shown in Fig. 6B, wild-type WBP-2 enhanced the transcriptional activity of PR in a hormone-dependent manner in transient transfection assays. Similarly, the WBP-2 proteins that contained mutations in either PPXY motif 1 or PPXY motif 2 were able to enhance PR activity, suggesting that PPXY motifs 1 and 2 of WBP-2 were nonessential for its coactivation functions (data not shown). In contrast, a WBP-2 protein that contained mutations in the cPPXY of WBP-2 was unable to coactivate the transcriptional activity of PR suggesting that this cPPXY (PY3mutant) of WBP-2 is required for its transcriptional function (Fig. 6B). Identical results were obtained with ER (data not shown). To confirm that the loss of coactivation function in the cPPXY mutant WBP-2 was not due to the loss of expression of the mutant WBP-2, we analyzed the expression of mutant WBP-2 by Western blot analysis. Fig. 6C demonstrates that both the wild-type and cPPXY mutant WBP-2 protein (PY3mutant) are expressed at approximately equal levels. To further ascertain that this loss of coactivation function of the cPPXY mutant WBP-2 is not due to its lack of interaction with the receptor, we also analyzed the interaction between wild-type WBP-2 and cPPXY mutant WBP-2 with ER in GST-pulldown assays. As shown in Fig. 6D, both wild-type WBP-2 and cPPXY mutant WBP-2 interact with ER in a hormone-dependent manner suggesting that loss of coactivation function of mutant WBP-2 is not due to loss of its interaction with receptor. Taken together these results demonstrate that *the cPPXY of WBP-2 protein act as an activation domain and is critical for its ability to enhance PR and ER-mediated transcription.*

***The Carboxy-Terminal PPXY Motif (cPPXY) of WBP-2 is an Integral Part of Its Intrinsic Activation Function**:* Authentic steroid hormone receptor coactivators often contain intrinsic transcription activation domains. To ascertain whether WBP-2 possesses an intrinsic transferable activation domain, wild-type WBP-2 and cPPXY mutant WBP-2 (coactivation deficient) were fused to GAL4-DNA binding domain. The ability of these fusion proteins to function as transcription activation domains was assayed using a GAL4-responsive reporter. In these experiments we utilized GAL4-SRC-1, a well characterized steroid hormone receptor coactivator as positive control. GAL4-WBP-2 (wild-type) was able to stimulate the transcriptional activity of the reporter gene to a greater extent then the vector containing only the

GAL4 DNA-binding domain, whereas the activity of the coactivation deficient mutant WBP-2 (cPPXY mutant) was significantly reduced compared to that of wild-type WBP-2 (Fig. 7). *These data suggest that cPPXY motif of WBP-2 is essential for its coactivation and intrinsic activation functions and most likely act as activation domain.*

II. Identification of the minimal receptor interaction motif of WBP-2

To identify the minimal receptor interaction (RID) motif of WBP-2 we proposed to use serial deletion mutants of WBP-2 in GST pull down assays. As proposed we are in the process of creating the deletion mutants. We do not envision any problems in identifying the RID of WBP-2 but the physiological impact of the RID fusion protein in the functional assay proposed in the original proposal has limited scope. *We deviated from the original SOW to explore the possibility that WBP-2 may bind to and regulate many WW-domain containing proteins in the cell as most of the known WW-domain containing proteins are regulated by other PY motif containing proteins. To further substantiate this claim we utilized a commercially available WW-domain array dot blot from Panomics InC.,*

Identification of WBP-2 Binding Proteins: Specific protein-protein interactions and multi-protein complexes are important for a multitude of cellular processes including gene transcription. As mentioned above WBP-2 via its PPXY motifs binds to proteins that contain WW-domain. WW-domains are found in both cytoplasmic and nuclear proteins, WW-domains containing proteins are involved in ubiquitination, nuclear signaling, cell cycle control, transcriptional regulation and the recruitment of signaling proteins. Since, WW-domains bind to the PPXY motif and WBP-2 has been shown to contain PPXY motifs, an important step toward characterizing coactivation function of WBP-2 is to identify to which particular WW-domain containing protein it bind, and hence determine the mechanism by which it act as a coactivator. In order to identify the possible WW-domain containing proteins that could interact with the WBP-2 protein, we utilized Panomics' TranSignal WW-domain Array (Panomics Inc., CA, USA). This array contains 67 different human WW-domains from 42 different proteins. The arrays are made using the recombinant conserved binding sites of individual WW-domains fused with GST. Proteins are affinity purified and immobilized onto a membrane. Each WW-domain is spotted in duplicate. In order to identify the WW-domain(s) that interact with WBP-2, the full-length wild-type WBP-2 protein containing flag tag was expressed in bacteria. Afterward, WBP-2 protein was purified on flag beads and incubated with TranSignal WW-domain Array membranes. The protein-protein interaction was visualized by using HRP-based chemiluminescence detection. Our screening data suggest that WBP-2 interacts with a wide variety of WW-domain containing proteins including YAP1, a transcriptional coactivator and WWOX1, a tumor suppressor (Fig. 8). In contrast, the cPPXY mutant WBP-2 protein was unable to bind to YAP1 (Fig9) and WWOX1 (data not shown). In this proposal, *we intend to dissect the molecular mechanism and significance of WBP-2:YAP1 interaction and WBP-2:WWOX1 interaction with relation to ER and PR function.* We also intend to examine *whether WWOX1 and YAP1 expression have opposite effects on ER and PR-dependent gene transcription.*

B. Role of WW-domain containing proteins in SHR function

The third specific aim is intricately related to the second aim so we pursuing both the aims simultaneously. As a consequence of the modification of aim two the scope of this aim is extended to include the newly indentified WW-domain containing proteins that may be involved in the mechanism of action of WBP-2 protein.

III. Generation of the WW-domain mutant of YAP

Our preliminary data strongly suggests that the WW-domain of YAP and the PY motif of WBP-2 are essential for their coactivation functions. We already generated and showed that the PY motif mutant of WBP-2 is lacking the coactivation function. To confirm that the WW-domain of YAP is also necessary for it coactivation activity we mutated the tryptophan (W) 199 to a phenylalanine (F) and tested its role in YAP's coactivation function.

The WW-Domain Containing Protein, YAP1, Modulates Progesterone Receptor Transcriptional Activity via the WBP-2 Protein*:* Since, WBP-2 binds to the WW-domain containing protein, YAP1 and YAP1 has been shown to be a transcriptional coactivator. Thus, we wanted to know whether YAP1 modulates steroid receptor-dependent target gene expression. To determine the role of YAP1 in steroid hormone receptor transactivation, HeLa cells were co-transfected with mammalian expression plasmids for the PR and ER receptors along with reporter plasmids containing their cognate hormone response element, with or without an expression vector for YAP1. *YAP1 alone did not affect PR-mediated transactivation either in the absence or presence of hormone.* In contrast, *when YAP1 was coexpressed with WBP-2 the hormone-dependent transcriptional activity of PR was significantly enhanced (~24-fold) (Fig. 9).* Similarly, YAP1 alone did not activate the ER-mediated transactivation but when co-expressed with WBP-2 enhanced ER-mediated transactivation (data not shown). This activity was higher than the observed coactivation with WBP-2 alone (Fig. 9). ***These data suggest that YAP1 can modulate the ligand-dependent transcriptional activity of PR and ER but only via WBP-2.*** The PPXY motifs of WBP-2 have been shown to interact with the WW-domain of YAP1. Since our data revealed that the cPPXY of WBP-2 was required for its coactivation function, we next asked whether the cPPXY of WBP-2 is required for YAP1 to function as a coactivator for ER and PR. When coexpressed together, wild-type WBP-2 and wild-type YAP1 greatly enhanced the transactivation function of PR (Fig. 9). In contrast, the cPPXY mutant WBP-2 and wild-type YAP1 also failed to enhance the transcriptional activity of PR (Fig. 9). Our data demonstrate that *the cPPXY motif of the WBP-2 protein is required for YAP1 to function as a transcriptional secondary coactivator.* This data is consistent with previously published reports that YAP1 stimulates gene transcription by binding to the PPXY motif of ErbB4 protein. In order to determine whether the WW-domain of YAP1 is required for its transcriptional coactivator function, a WW-domain mutant (W199F) was utilized. This YAP1 mutant has been shown to be inactive in its ability to bind to PPXY motif. When coexpressed together, wild-type WBP-2 and WW-domain mutant YAP1, the WW-domain mutant YAP1 fail to act as a coactivator (data not shown). These data suggest that *WW-domain of YAP1 is required for it to function as a transcriptional coactivator.* In summary, our preliminary data *substantiate the role of WBP-2 (contains*

PPXY motif) and YAP1 (contains WW-domain) in female steroid hormone receptor function. Based on our data, we postulate that *the cPPXY motif of WBP-2 binds to the WW-domain of YAP1 and recruits YAP1 to the target gene promoter by interacting with receptor. When the receptor-WBP-2-YAP1 complex is recruited to hormone responsive promoters, it acts at one of the many substeps required to modulate the transactivation functions of ER and PR- responsive target genes.*

IV. Role of the WW-domain containing protein, WWOX1 in WBP-2:YAP mediated ER and PR signaling

As mentioned above, we have also identified another WW-domain containing protein, WWOX1 as a WBP-2 interacting protein in our WW-domain array. WWOX1 interacts with WBP-2 via its first WW-domain. WWOX1 was originally cloned as a putative tumor suppressor gene and it has been suggested that WWOX1 may play an important role in hormone regulated cancers [32, 33, 70, 78, 79]. Previously, it has been shown that WWOX1 interacts with PPXY motif containing proteins, p73 and AP-2 and suppresses their transcriptional activities. Most recently, it has been shown that WWOX1 interacts with another PPXY motif containing protein, ErB-4 and compete with YAP1 for ErB-4 binding and suppress the coactivation functions of YAP1 [74]. Since, WWOX1 and YAP1 have the similar tandem WW-domains and both interact with common protein, WBP-2; we ask whether WWOX1 and YAP1 expression have opposite effects on ER and PR-dependent gene transcription. To determine the role of WWOX1 in steroid hormone receptor transactivation, MCF-7 cells were co-transfected with ER-responsive reporter plasmid along with expression vectors for either WBP-2, YAP1, and WWOX1, WBP-2 and WWOX1 or WBP-2, YAP1 and WWOX1. As shown before, WBP-2 coactivates the transactivation functions of ER. Furthermore, YAP1 and WWOX1 alone had no significant effect on ER function. But when YAP1 was coexpressed with WBP-2 the hormone-dependent transcriptional activity of ER was synergistically enhanced (Fig. 10). In contrast, expression of WWOX1 significantly reduced the coactivation functions of WBP-2. Similarly, WWOX1 also significantly suppressed WBP-2-YAP1-mediated transcriptional activities of ER and PR in a dose-dependent manner (Fig. 10). To determine whether the WW-domain of WWOX1 is required for its transcriptional suppression function, a WW-domain mutant was utilized in which the tryptophan 33 within WW-domain 1 was mutated to arginine. This WWOX1 mutant has been shown to be inactive in its ability to bind to PPXY motif. When coexpressed along with wild-type WBP-2 and wild-type YAP1, this mutant had no significant effect on WBP-2 and YAP1's coactivation functions (Data not shown). Identical results were obtained with PR (data not shown). These data indicate that *the coactivation functions of WBP-2 and YAP1 are suppressed by WWOX1, suggesting that WWOX1 may regulates the transactivation functions of ER and PR by antagonizing the functions of WBP-2 and YAP1.*

C. Expression analysis of endogenous WBP-2 protein

In light of the recent modifications to the original proposal we have accommodated and updated the first aim of the proposal. In the original proposal we intended to analyze only the expression profile of WBP-2 in various cancer cell lines and human breast tissue arrays. Our current understanding is that YAP1 and WWOX1 may play vital roles in the regulations and function of WBP-2, furthermore YAP1 has been shown to be amplified in various breast cancers and intriguingly,

WWOX1 has been show to be a potent tumor suppressor [74]. Given these interesting facts we propose to analyze the expression of WBP-2, YAP1 and WWOX1 in correlation with the ER and PR expression status of various breast cncer cell lines as well as many breast tumor-arrays which may shed some light on the complicity of WBP-2 function and the possible roles of YAP1 and WWOX1 in breast cancers.

Key Research Accomplishments

- Establishing that WBP-2 is a coactivator of ER and PR
- The PY motif of WBP-2 is essential for its coactivation function
- Identification of YAP1 and WWOX1 as WBP-2 interacting proteins
- YAP1 acts as transcriptional secondary coactivator of ER and PR that is strictly dependent of WBP-2
- WW-domain of YAP and the PY motif of WBP-2 are essential for their coactivation activities
- WWOX1 may act as a transcriptional repressor of WBP-2

Reportable Outcomes

1. The first part of the this work has been published "Dhananjayan, S.C., et al., *WW Domain Binding Protein-2, an E6-Associated Protein Interacting Protein, Acts as a Coactivator of Estrogen and Progesterone Receptors.* Mol Endocrinol, 2006. **20**(10): p. 2343-2354" (Appendix 2).

2. The second part of this report has been accepted to be presented at the Annual Endocrine Society Meeting, ENDO 2007 (June 2-5th), in Toronto, Canada (Appendix 3).

Conclusions

Our data demonstrates that WBP-2 is recruited onto the hormone responsive promoters in the presence of hormone and it specifically enhances the transactivation functions of PR and ER. Our data also demonstrates that WBP-2 contains an intrinsic activation domain and the cPPXY of WBP-2 is essential for its coactivation and intrinsic activation functions. Our preliminary data also demonstrates that the WBP-2 binding protein, YAP1 enhances PR and ER transactivation but YAP1's coactivation function is absolutely dependent on WBP-2. Furthermore, cPPXY motif of WBP-2 and WW-domain of YAP1 is required for YAP1 to work as a transcriptional coactivator. Additionally, our data also indicate that the coactivation functions of WBP-2 and YAP1 are suppressed by WWOX1, suggesting that WWOX1 may regulates the transactivation functions of ER and PR by antagonizing the functions of WBP-2 and YAP1. Taken together our data established the role of WBP-2 and YAP1 as coactivators and WWOX1 as a repressor for ER and PR transactivation pathways.

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Appendices

1. Figures
2. 1 Published Manuscript and 1 Abstract

Appendix 1

Figures 1-11

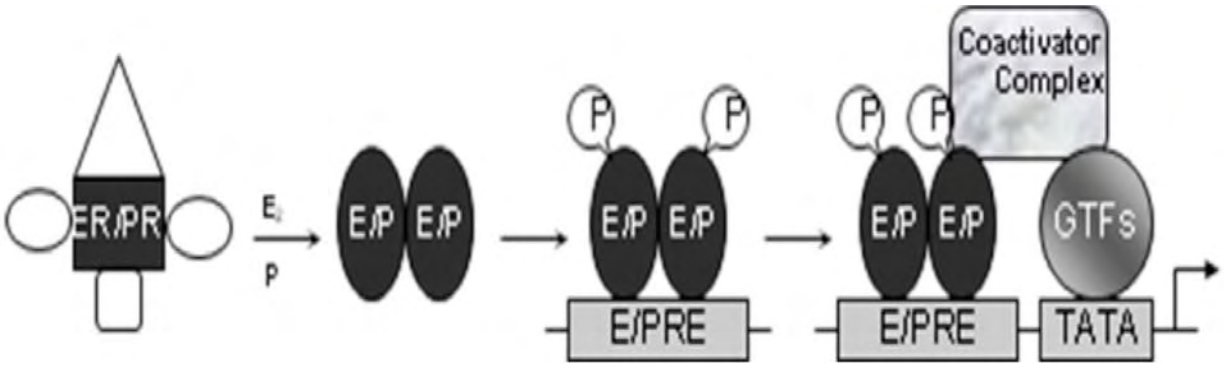


Fig. 1: A model of transcriptional coactivation of ER and PR.

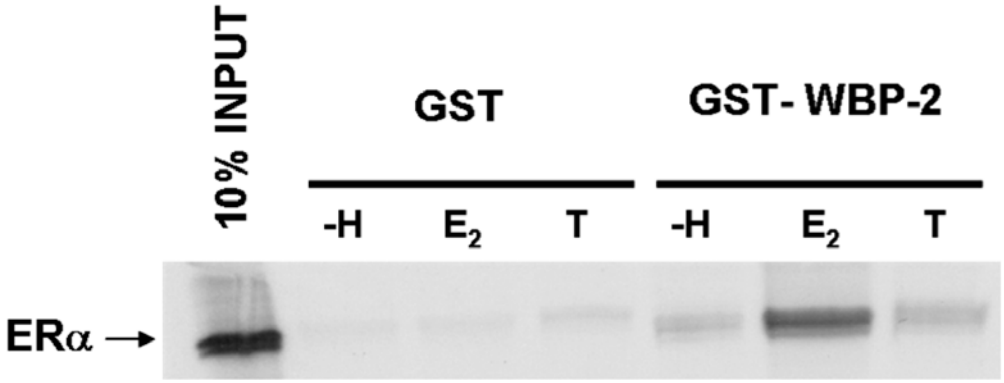


Fig. 2A: WBP-2 interacts with ER

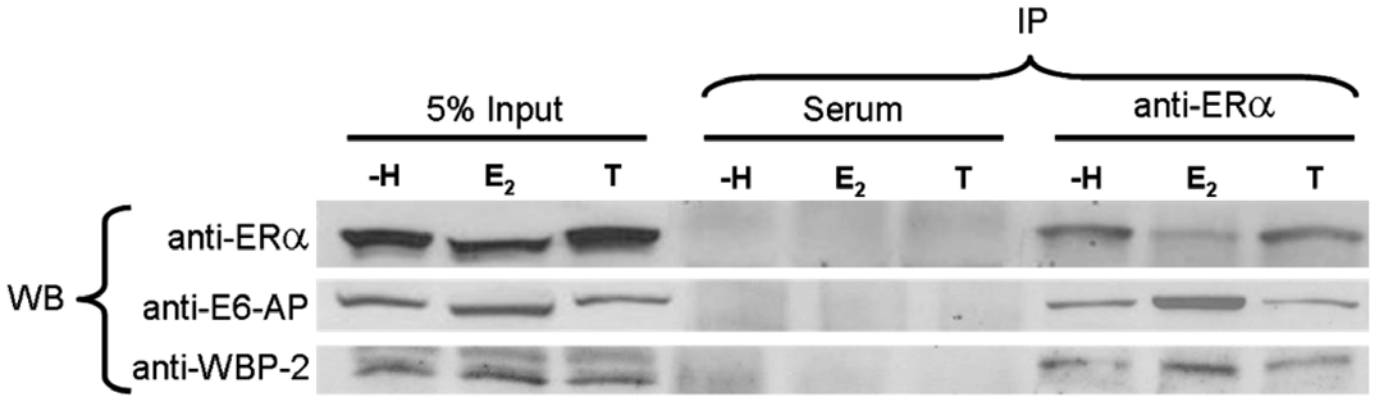


Fig. 2B: WBP-2 and E6-AP co-precipitates with inter-acts with ER

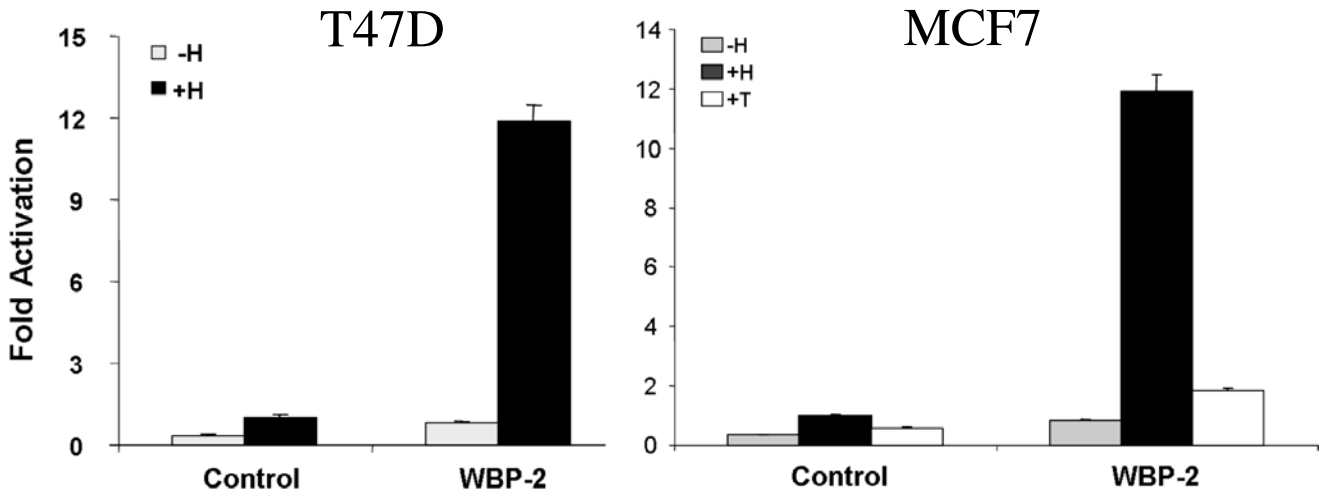


Fig. 3A: WBP-2 specifically coactivates ER and PR

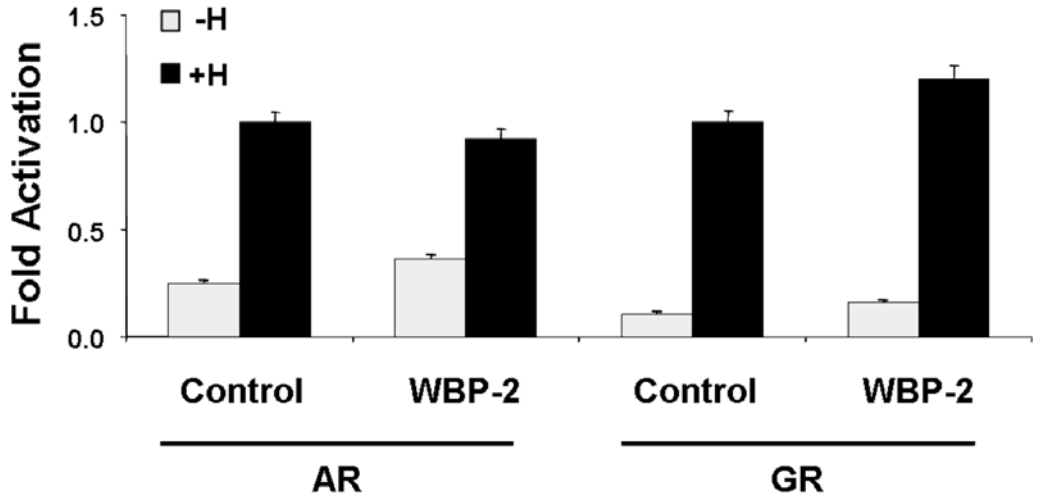


Fig. 3B: WBP-2 has not effect on AR and GR transactivaion

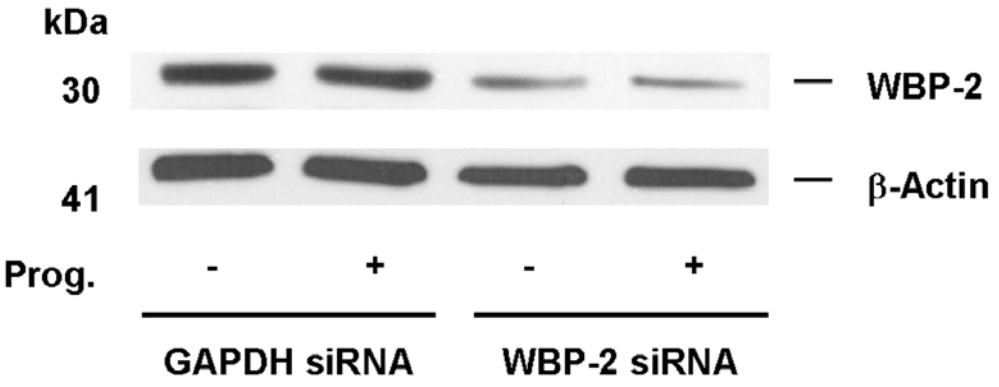
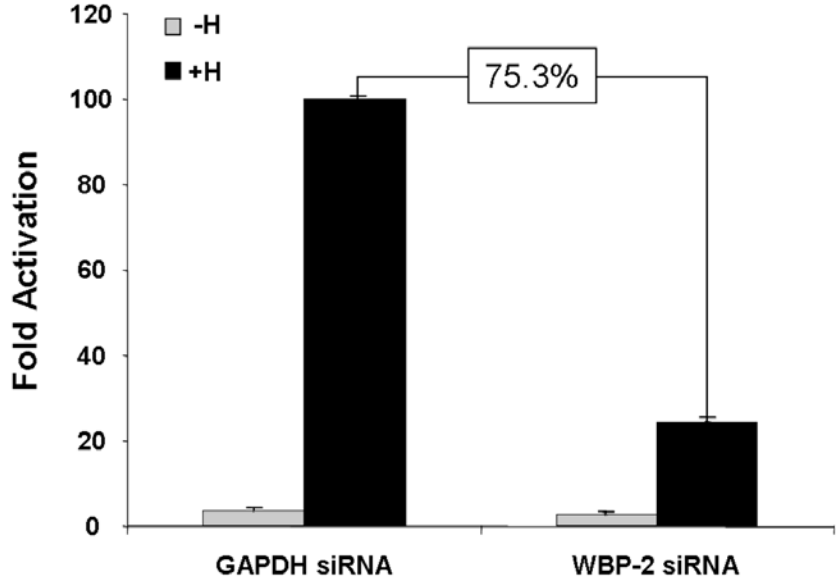
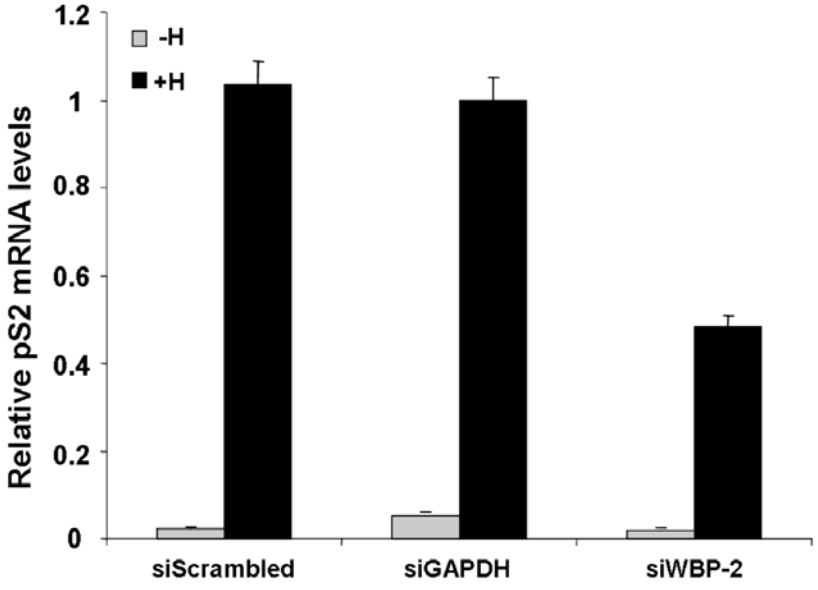


Fig. 4A: siRNA mediated specific knock-down of WBP-2





gene activation

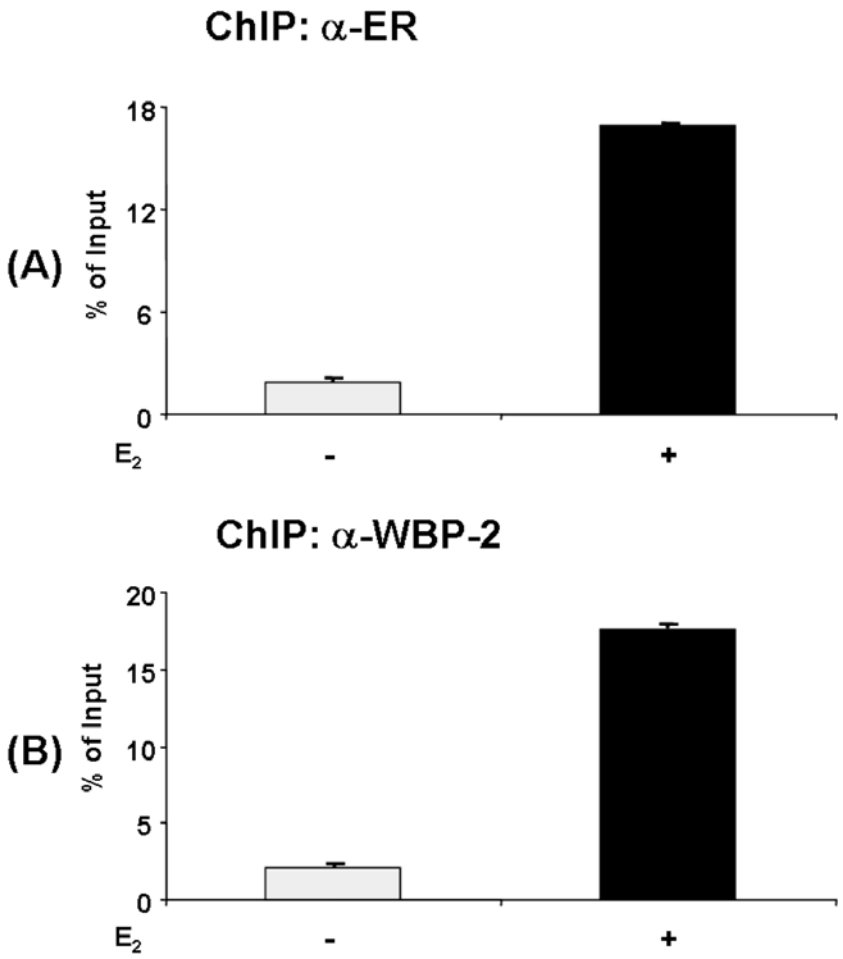


Fig. 5: WBP-2 is recruited to pS2 (ER target gene) promotor

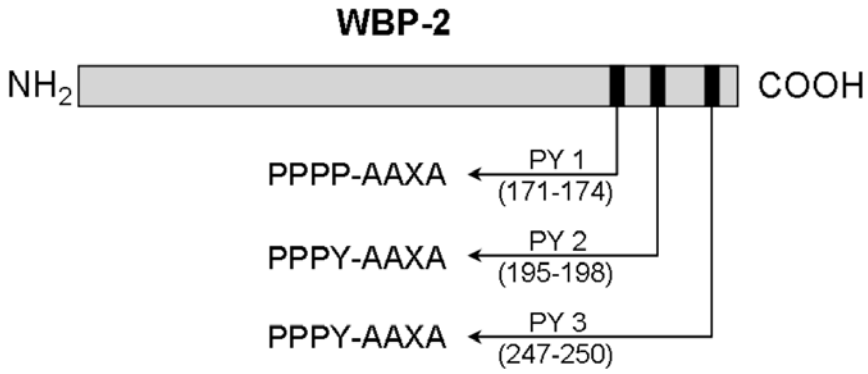


Fig. 6A: Schematic showing WBP-2 mutations

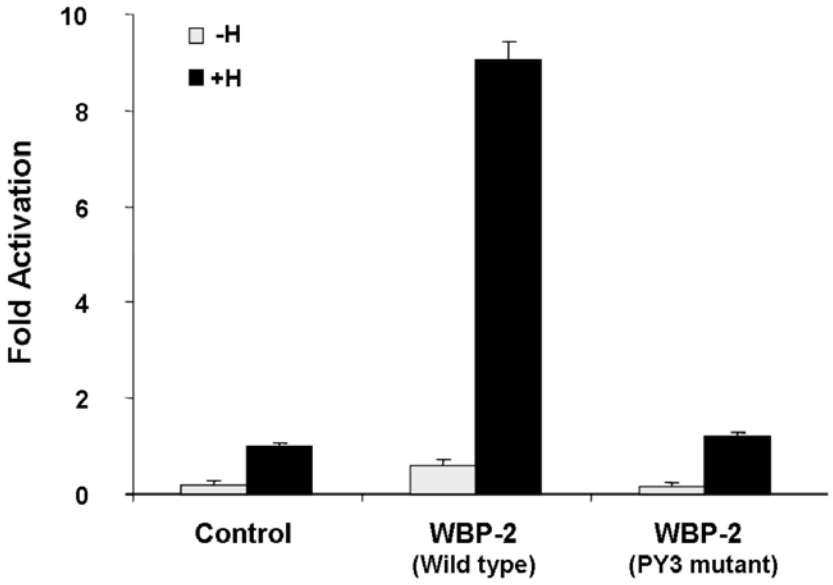


Fig. 6B: PY motif of WBP-2 is required for its coactivation function

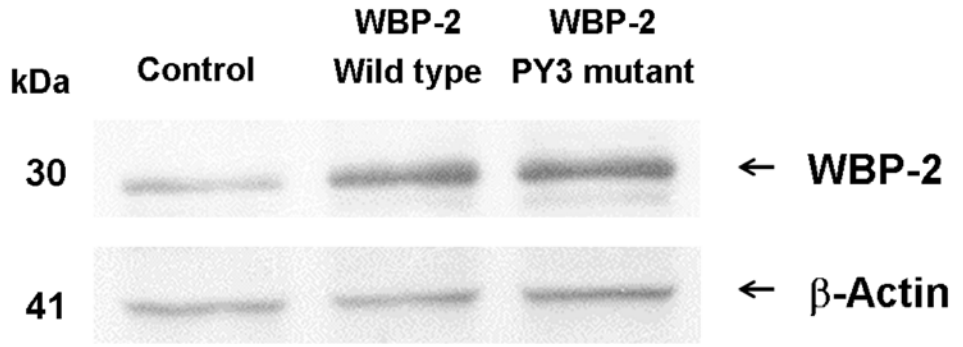


Fig. 6C: WBP-2 mutant is functionally inactive

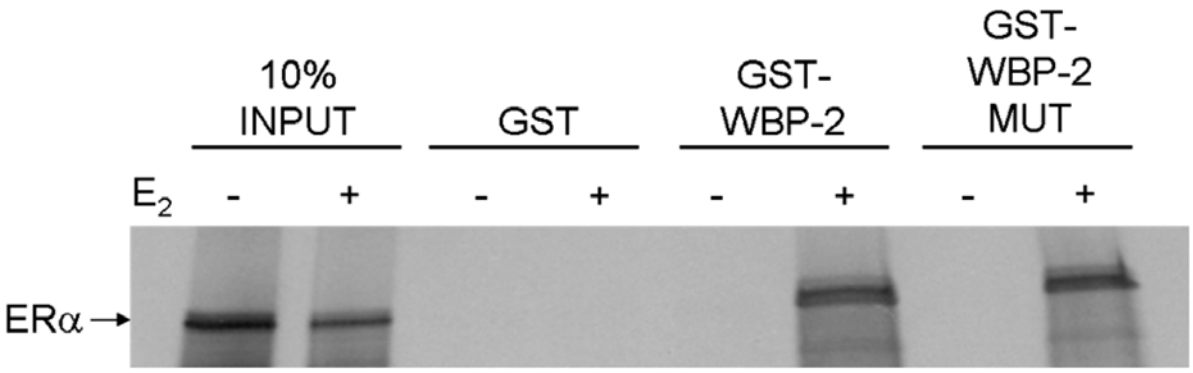


Fig. 6D: WBP-2 mutant can interact with ER

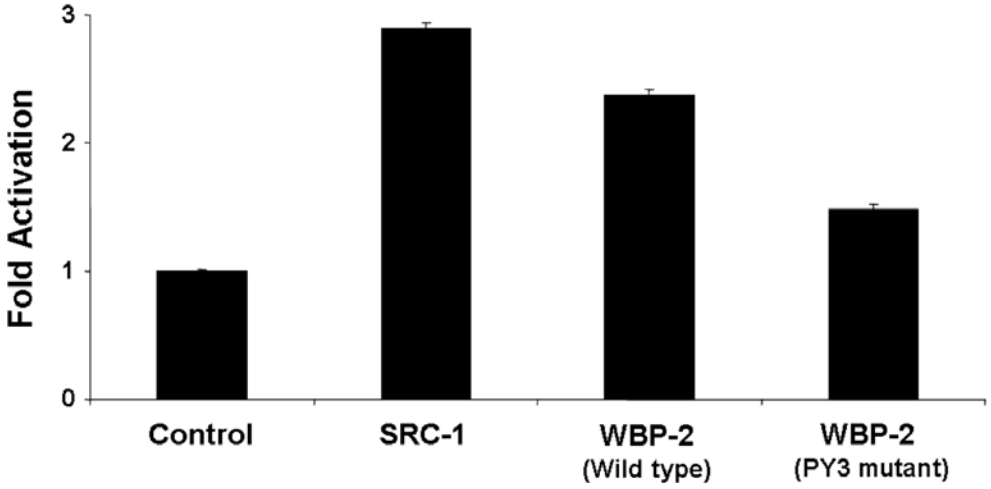


Fig. 7: WBP-2 has intrinsic activation domain

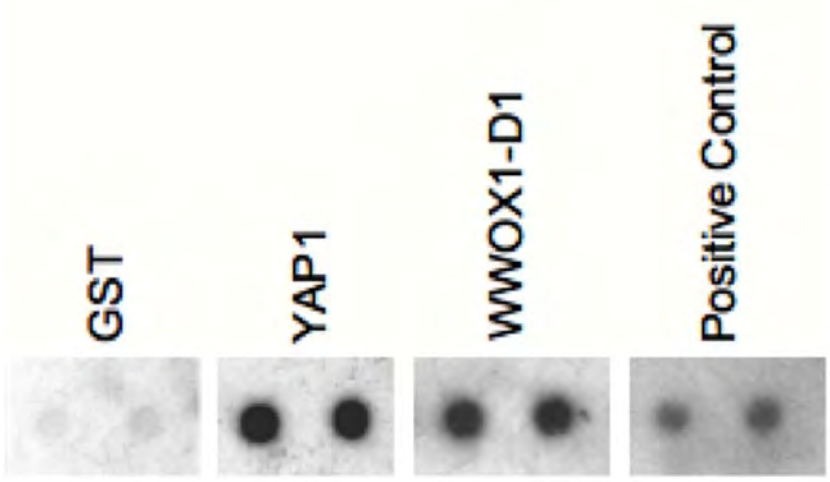


Fig. 8: WBP-2 interacting proteins

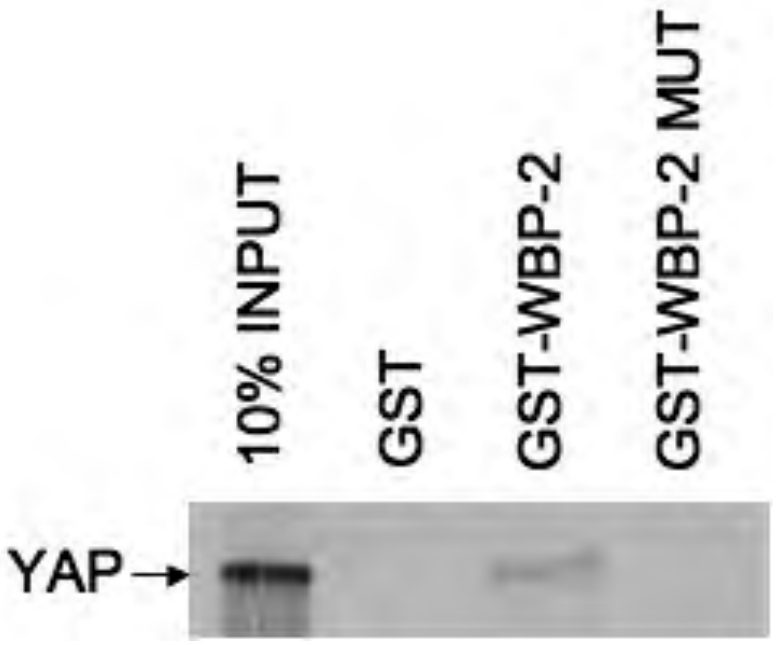


Fig. 9: PY motif of WBP-2 is essential for interaction with YAP1

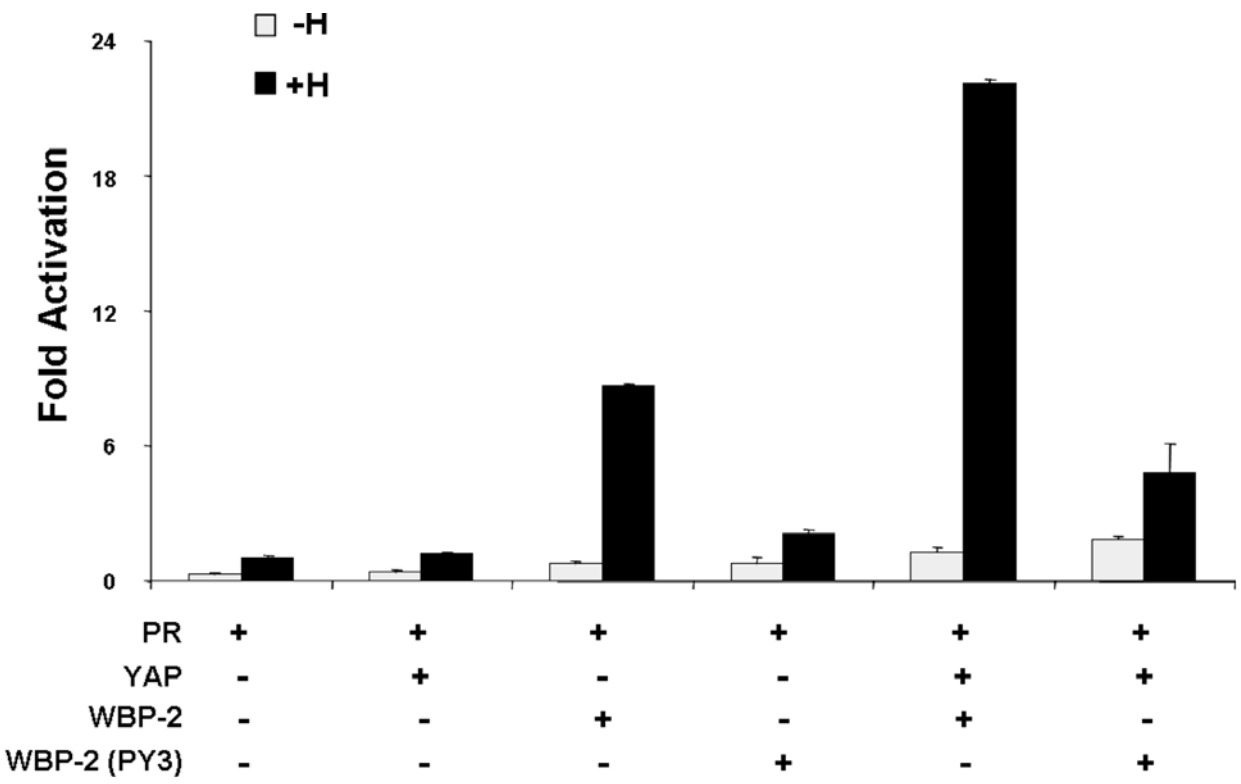


Fig. 10: YAP coactivation function is dependent on the PY motif of WBP-2

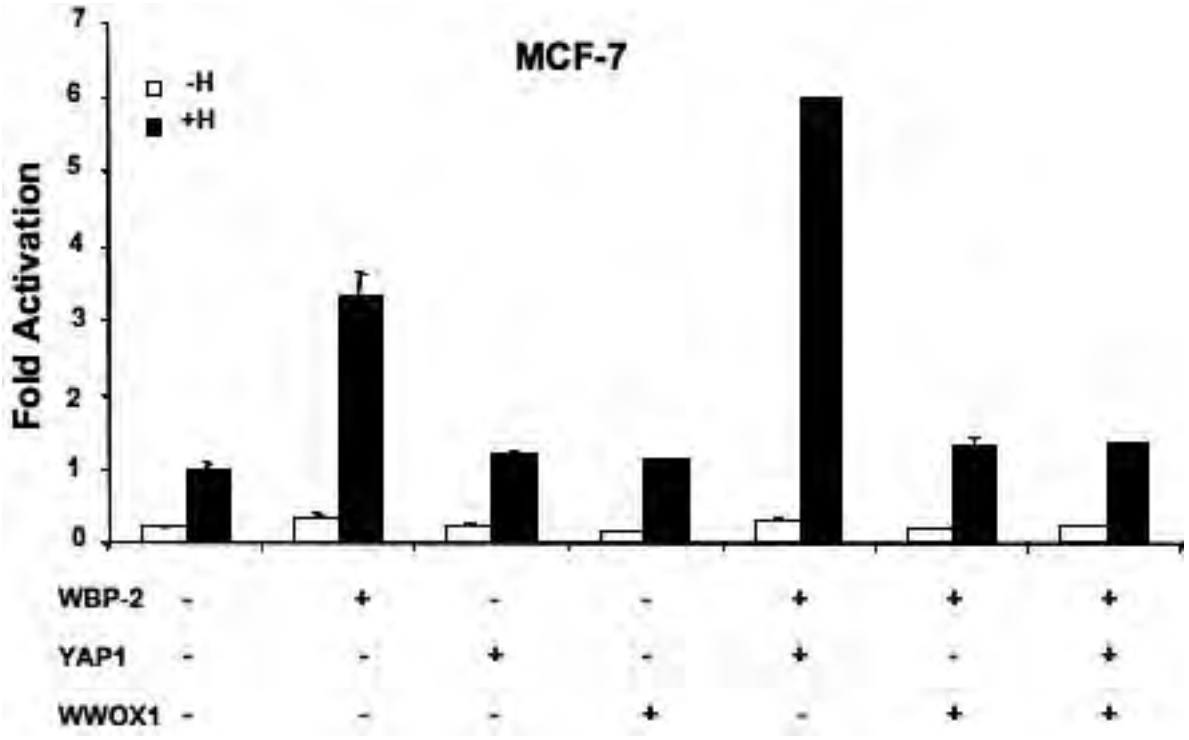


Fig. 11: WWOX1 represses the coactivation functions of WBP-2 and YAP1

Appendix 2

1 Abstract

1 Manuscript

The Endocrine Society's 89th Annual Meeting

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Title: Oncogenic WW-domain containing oxidoreductase, negatively regulates the coactivation functions of WW-domain binding protein-2 and yes-associated protein 1

Sarath C Dhananjayan1, Sivapriya Ramamoorthy1 and Zafar Nawaz1. 1Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida, United States, 33136.

Steroid hormone receptors (SHR) are intracellular ligand activated transcription factors that function by stimulating target gene transcription in response to their cognate lipophilic ligand. Previously, we have demonstrated that WW-domain binding protein-2 (WBP-2) acts as coactivator for estrogen receptor (ER) and progesterone receptor (PR). We showed that the carboxy terminal polyproline (PY) motif of WBP-2 is required for it coactivation function (1). Given all this we are interested is elucidating the molecular mechanism of action of WBP-2. Since PY motifs specifically interact with WW-domains we were interested in identifying the WW-domain containing proteins that interact with WBP-2. We tested the interaction of full-length WBP-2 with the WW-domain array (Panomics, Inc.) in a dot bolt analysis. WW domain-containing oxidoreductase (WWOX) and yes-associated protein 1 (YAP1) were identified in the screen. WBP-2 has been shown to interact with YAP1, which contains a consensus WW-domain and is a well-known transcription activator. We have also showed that YAP1 acts as a transcriptional secondary coactivator of ER and PR as its coactivation function is strictly dependent on WBP-2 (1). However, WWOX is an oncogenic protein that is frequently lost in ER positive breast cancer and has been shown to a transcriptional suppressor. Further, WWOX has been shown to complete with YAP in binding to PY motif containing proteins. In this current study, we report that when coexpressed WWOX negatively regulates the WBP-2:YAP mediated coactivation of ER and PR

transactivation pathways. Our results show that WBP-2 and WWOX interact in vivo in a dose dependent manner. We also show that WWOX competes with YAP1 for WBP-2 binding and retains it in the cytoplasm. Since WBP-2 acts as a coactivator of ER and PR signaling in the nucleus WWOX mediated cytoplasmic retention of WBP-2 distrupts its coactivation function. Taken together our data represents a novel mechanism of regulation of coactivator (WBP-2) proteins by an oncogenic protein (WWOX) based on this we postulate that WBP-2 via its cPPXY motif interacts with WW-domain of YAP1 and then WBP-2:YAP1 complex is recruited onto the hormone responsive promoter and regulate target gene transcription. We further postulate that WWOX physically competes with YAP1 for cPPXY motif of WBP-2 and interferes with its ability to bind to WBP-2 and blocks WBP-2 and YAP1's coactivation functions.

References: (1) Dhananjayan, S.C., et al., WW Domain Binding Protein-2, an E6-Associated Protein Interacting Protein, Acts as a Coactivator of Estrogen and Progesterone Receptors. Mol Endocrinol, 2006. 20(10): p. 2343-2354.

Sources of Support: FY05 Predoctoral Traineeship Award DOD **BC050753** awarded to SCD and Braman Family Breast Cancer Institution startup funds to ZN.

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WW Domain Binding Protein-2, an E6-Associated Protein Interacting Protein, Acts as a Coactivator of Estrogen and Progesterone Receptors

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Department of Biochemistry and Molecular Biology (S.C.D., S.R., O.Y.K., A.I., J.Su., J.Sl., Z.N.), University of Miami School of Medicine, Miami, Florida 33136; and Department of Molecular and Cellular Biology (B.W.O.), Baylor College of Medicine, Houston, Texas 77030

WW domain binding protein-2 (WBP-2) was cloned as an E6-associated protein interacting protein, and its role in steroid hormone receptors functions was investigated. We show that WBP-2 specifically enhanced the transactivation functions of progesterone receptor (PR) and estrogen receptor (ER), whereas it did not have any significant effect on the androgen receptor, glucocorticoid receptor, or the activation functions of p53 and VP-16. Depletion of endogenous WBP-2 with small interfering RNAs indicated that WBP-2 was required for the proper functioning of PR and ER. We also demonstrated that WBP-2 contains an intrinsic activation domain. Moreover, chromatin immunoprecipitation assays demonstrate the hormone-dependent recruitment of WBP-2 onto an estrogen-responsive promoter. Mutational analysis suggests that one of three polyproline (PY) motifs of WBP-2 is essential for its coactivation and intrinsic activation functions. We show that WBP-2 and E6-associated protein each enhance PR function, and their effect on PR action are additive when coexpressed, suggesting a common signaling pathway. In this study, we also demonstrate that the WBP-2 binding protein, *Yes* kinase-associated protein (YAP) enhances PR transactivation, but YAP's coactivation function is absolutely dependent on WBP-2. Taken together, our data establish the role of WBP-2 and YAP as coactivators for ER and PR transactivation pathways. (*Molecular Endocrinology* 20: 2343–2354, 2006)

STEROID HORMONES REGULATE various biological processes via their cognate receptors, which are comprised of a superfamily of structurally related intracellular ligand-activated transcription factors (1, 2). In the absence of hormones, these receptors are transcriptionally inactive and are bound to cellular chaperone proteins. Upon ligand binding, the receptors undergo a conformational change, resulting in their dissociation from cellular chaperones, dimerization, and phosphorylation. Ligand-bound receptor dimers bind to enhancer elements of target genes and subsequently recruit coactivators and general transcription factors to form a preinitiation complex that culminates in target gene transcription (3–7).

First Published Online June 13, 2006

Abbreviations: AR, Androgen receptor; ChIP, chromatin immunoprecipitation; E2, estradiol; E6-AP, E6-associated protein; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; GST, glutathione-*S*-transferase; PR, progesterone receptor; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; SRC, steroid receptor coactivator; WBP-2, WW domain binding protein-2; YAP, *Yes* kinase-associated protein.

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Nuclear hormone receptor coactivators are molecules that interact with activated receptors and stimulate receptor-mediated transcription of target genes (8–11). The most widely studied coactivators include members of the p160 family of coactivators; steroid receptor coactivator-1 (SRC-1), SRC-2 [transcription intermediary factor-2 (TIF-2)/glucocorticoid receptor interacting protein-1 (GRIP-1)], SRC-3 [p/CIP; p300/CBP interacting protein/activator of thyroid and retinoid acid receptors (ACTR)/amplified in breast cancer-1 (AIB-1)/retinoid acid receptor coactivator-3 (RAC-3)/thyroid receptor activator molecule-1 (TRAM-1)], the cAMP response element-binding protein-binding (CREB) protein (CBP)/p300 family coactivator-associated arginine methyltransferase (CARM-1), and E6-associated protein (E6-AP) (11–18).

We have previously reported the cloning and characterization of E6-AP as a novel dual-function steroid hormone receptor coactivator. Recently, we demonstrated that the E2 ubiquitin-conjugating enzyme, UbcH7, acts as a coactivator of steroid hormone receptors (19). It has been demonstrated that coactivator proteins form multiprotein complexes to efficiently regulate target gene transcription (15). In this study, our focus was to identify proteins that interact with E6-AP and modulate steroid hormone receptor functions. To identify proteins that interact with E6-AP, we performed a yeast two-hybrid screen using E6-AP mu-

tant (E6-AP-C833S) defective in ubiquitin-ligase function as bait so as to avoid degradation of the prey during the process. WW domain binding protein-2 (WBP-2) was identified as an E6-AP interacting protein. WBP-2 was previously shown to interact with human *Yes* kinase-associated protein (YAP) via the WW domain of YAP protein (20). The WW domain is characterized by 35–40 semiconserved amino acids, which are involved in protein-protein interaction (21). WBP-2 interacts with the WW domain via a short proline-rich motif (PPXY) with the consensus sequence of four consecutive prolines followed by tyrosine (22). It has been speculated that WBP-2 plays a role in transcription, but its exact function in transcription has not been defined. Additionally, it has been suggested that YAP may regulate transcription (23) by acting as a coactivator for several transcription factors including members of Runx2 family (24) and TEAD/TEF family (25), the proapoptotic protein p73 (26, 27), and is involved in ErbB4 signaling (28).

In the present study, we describe a role for WBP-2 and YAP in steroid hormone receptor functions. We show that WBP-2 physically interacts with E6-AP and certain steroid hormone receptors. We demonstrate that WBP-2 specifically modulates the hormone-dependent transcriptional activities of estrogen receptor (ER) and progesterone receptor (PR). Moreover, depletion of endogenous WBP-2 protein with small interfering RNA (siRNA) significantly reduces the transactivation potential of steroid receptors. Our data suggest that the carboxyl-terminal PPXY motif of WBP-2 is required for its coactivation function. Coexpression of WBP-2 and E6-AP enhances steroid receptor transactivation additively. Furthermore, YAP, which has been shown to be physically associated with WBP-2, also is able to enhance receptor function. However, the YAP coactivation function is strictly dependent on the coexpression of functional WBP-2. Together, our results demonstrate the role of WBP-2 as a potent coactivator for a subset of steroid receptors.

Results

Isolation and Characterization of WBP-2 as an E6-AP Interacting Protein

Yeast two-hybrid screening system was used to isolate cDNA clones that encode E6-AP interacting proteins. Because E6-AP could activate the degradation of its binding proteins, a catalytically inactive form of E6AP (E6-AP-C833S) was used as bait to avoid potential degradation of interacting proteins. E6-AP (C833S) was fused in-frame with the Gal4 DNA-binding domain and introduced into *Saccharomyces cerevisiae*. The prey cDNA library, fused to the Gal4 activation domain, was derived from human brain cells. We isolated 12 colonies with cDNAs encoding proteins that strongly interacted with E6-AP. Surprisingly, all 12 colonies contained identical cDNAs. A sequence similarity search in the GenBank database revealed that all colonies encoded the carboxyl terminus of WBP-2.

We subsequently verified that WBP-2 interacts with E6-AP both *in vivo* and *in vitro*. As shown in Fig. 1A, in a yeast two-hybrid assay, WBP-2 strongly interacts with E6-AP. To further confirm that the WBP-2 and E6-AP interaction observed in the yeast two-hybrid assay is due to direct physical association of WBP-2 with E6-AP, we performed glutathione-*S*-transferase (GST) pull-down assays. In this assay, WBP-2 interacted directly with E6-AP (Fig. 1B).

WBP-2 Interacts with Estrogen and Progesterone Receptors

WBP-2 interacts with E6-AP and E6-AP has been shown to act as coactivator for certain nuclear and steroid hormone receptors. To determine whether WBP-2 interacts with steroid hormone receptors, we assayed WBP-2 binding to ER and PR in GST pull-down assays. Figure 2A shows strong WBP-2 interaction with full-length ER in the presence of estradiol and reduced binding in the absence of hormone and presence of antiestrogen (tamoxifen). Under similar conditions, WBP-2 was unable to interact with control

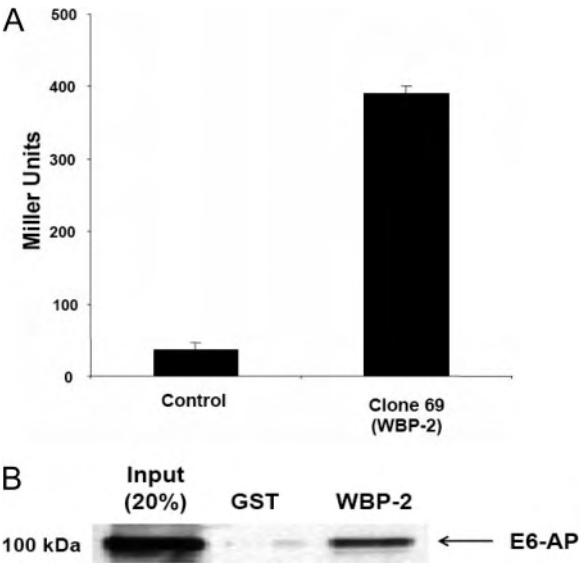

Fig. 1. Yeast Two-Hybrid Screen for E6-AP Interacting Proteins

A, Yeast cells were transformed with bait plasmid pGAD10-E6-AP (C833S) and prey brain cDNA library, fused to the Gal4 activation domain. The β-galactosidase activity (Miller units) of clone 69 (WBP-2) was determined. B, *In vitro* WBP-2 interacts with E6-AP. *In vitro* translation of E6-AP was performed in the presence of [^{35}S]methionine using the transcription and translation kit (Promega). GST-WBP-2 and GST alone (control) were expressed in *E. coli* and purified on glutathione-Sepharose beads. The purified and glutathione-bound GST (control) or GST-WBP-2 was incubated with *in vitro*-translated E6-AP. WBP-2-bound E6-AP was analyzed by autoradiography with 20% of *in vitro*-translated E6-AP as input.

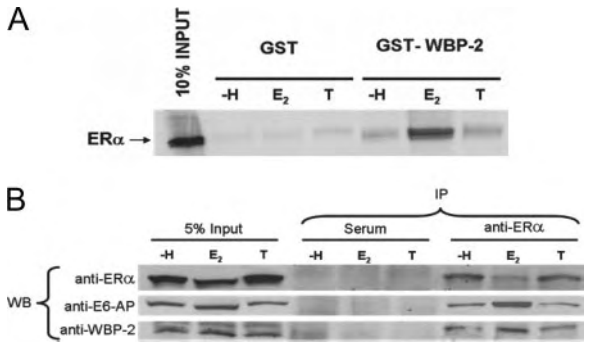

Fig. 2. WBP-2 Interacts with ER

A, Interaction of WBP-2 with ER was determined in a GST pull-down assay. ER was radiolabeled with ^{35}S by the *in vitro* transcription and translation kit. The labeled ER protein was then incubated overnight at 4 C with *E. coli* expressed GST alone (control) or GST-WBP-2 bound to beads either in the absence of hormone (−H), or presence of E2 or tamoxifen (T). Bound proteins were analyzed by SDS-PAGE and autoradiography with 10% of *in vitro*-translated ER as input. B, *In vivo* WBP-2 interacts with E6-AP and ER. MCF-7 cells were treated with either no hormone (−H), E2, or tamoxifen (T) for 24 h, and cells were lysed with RIPA lysis buffer. Lysates were clarified with protein A-Sepharose beads and incubated with either anti-ER antibody or rabbit preimmune serum (Alpha Diagnostic). The lysates were precipitated with protein A-Sepharose beads that bind to rabbit IgGs followed by electrophoresis. Five percent of the lysates were used as input and analyzed by Western blot with either anti-ER, anti-E6-AP, or anti-WBP-2 antibodies.

GST protein. Similarly, WBP-2 and PR interaction was observed in the presence of progesterone, whereas only minimal binding was observed in the absence of hormone and presence of antiprogestin (RU486) (data not shown). These findings indicate that WBP-2 can interact with ER and PR in the presence of hormone in *in vitro*.

WBP-2 and E6-AP Coprecipitate with ER in Cellular Lysates

Although WBP-2 could bind to both E6-AP and ER *in vitro*, we wanted to determine whether all three proteins are present in a complex *in vivo* in the presence of hormone. Immunoprecipitation assays using MCF-7 cell lysates treated with vehicle (ethanol), estradiol, or antiestrogen (tamoxifen) showed that both WBP-2 and E6-AP strongly interact with ER in the presence of estradiol (Fig. 2B). However, a weaker interaction of E6-AP, WBP-2, and ER was observed in the absence of hormone and tamoxifen. This observation is in agreement with our *in vitro* assays, and furthermore, it suggests that WBP-2, E6-AP, and ER form a complex.

WBP-2 Selectively Modulates the Transcriptional Activities of Progesterone and Estrogen Receptors

Because E6-AP acts as a coactivator for nuclear hormone receptors and WBP-2 physically associates with E6-AP and steroid hormone receptors, we next tested whether WBP-2 regulates receptor-dependent activation of target gene expression using transient transfection assays in HeLa cells. HeLa cells were cotransfected with mammalian expression plasmids containing one of the following receptors, PR, ER, glucocorticoid (GR) and androgen (AR) receptors along with the reporter plasmids driven by their cognate hormone response element. These assays were carried out with or without coexpression of an expression vector for WBP-2. WBP-2 had little effect on the transactivation functions of PR and ER in the absence of hormone. However, WBP-2 significantly enhanced (~4- to 5-fold) the hormone-dependent transcriptional activities of PR and ER (Fig. 3A). WBP-2 had only minimal effect on the transcriptional activities of GR and AR both with and without their cognate ligands (Fig. 3B). Similarly WBP-2 had only negligible effect on other nuclear receptors like retinoic acid receptor-α and thyroid receptor (data not shown). These data suggest that WBP-2 selectively modulates the ligand-dependent transcriptional activities of ER and PR. Because HeLa cells express the viral E6 protein that could functionally interact with WBP-2 via E6-AP, we wanted to assay the coactivation function of WBP-2 in cells that lack E6 expression. As shown in Fig. 3C, WBP-2 stimulated the hormone-dependent transcriptional activity of PR in the E6-negative T47D cells. Similarly, WBP-2 could also stimulate the hormone-dependent transcriptional activity of ER in the E6-negative MCF-7 cells. Furthermore, WBP-2 has no significant effect on the transcriptional activity of ER in the presence of antiestrogen (tamoxifen), suggesting that WBP-2 only enhance the hormone-dependent transcriptional activity of receptor (Fig. 3D). These data also indicate that the coactivation function of WBP-2 is not dependent on the E6 protein. This is consistent with our previously published data, indicating that the coactivation function of E6-AP is not dependent on the viral E6 protein (16).

To further examine whether WBP-2 selectively modulates the transcriptional activities of ER and PR, we investigated how WBP-2 affected the transcriptional activities of unrelated transcription factors, p53 and the VP-16. As shown in Fig. 3E, the transcriptional activities of p53 and the VP-16 activation domain were not affected by exogenously expressed WBP-2. These observations suggest that WBP-2 preferentially modulates the hormone-dependent transcriptional activity of a subset of steroid hormone receptors.

Depletion of Endogenous WBP-2 Levels Reduces the Transcriptional Activity of PR and ER

To confirm that WBP-2 is indeed required for PR activation, cellular WBP-2 expression was down-regulated in HeLa cells using WBP-2-directed siRNA. HeLa cells were transiently transfected with siRNAs directed against either WBP-2 or against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control, together

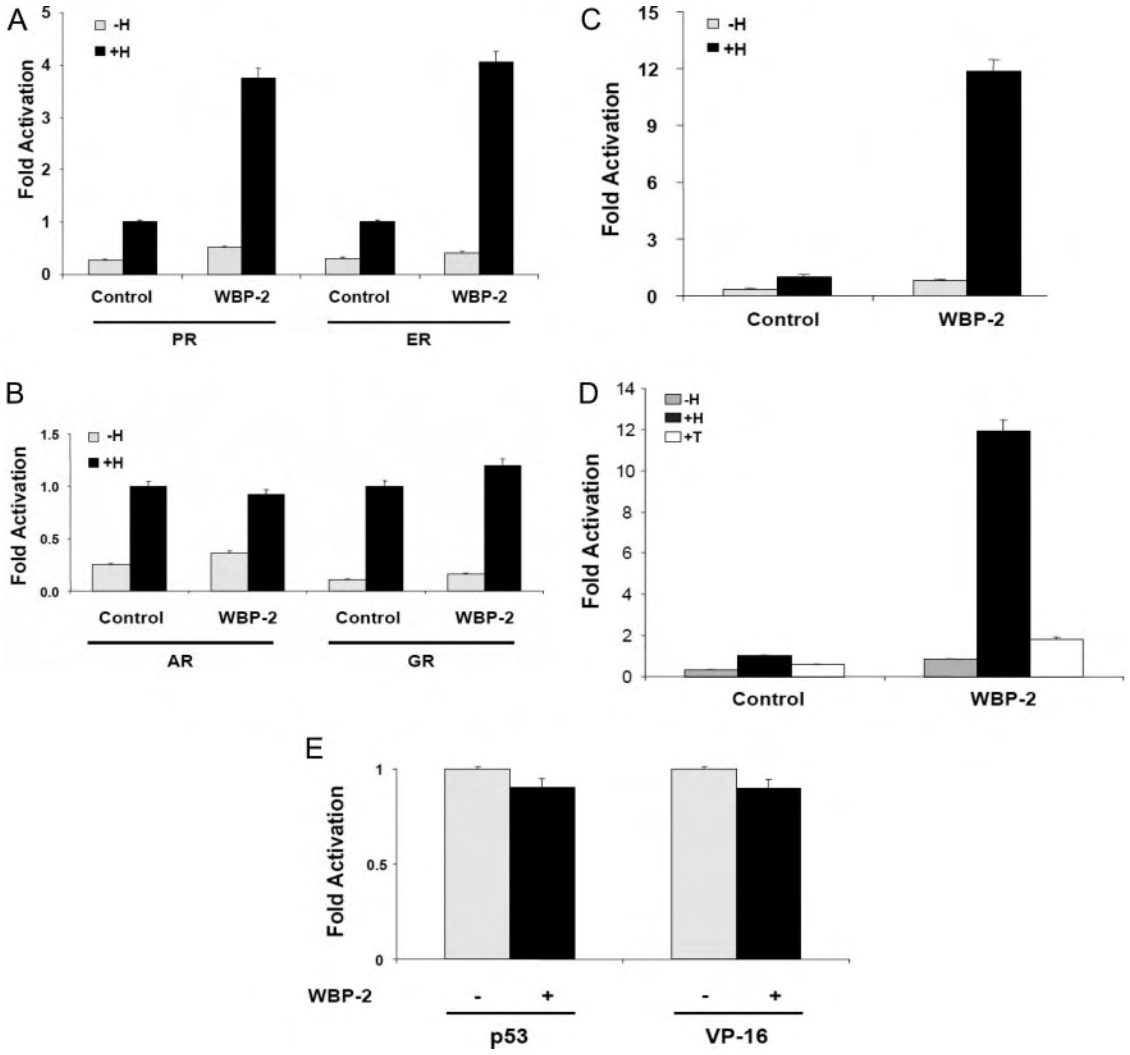

Fig. 3. WBP-2 Specifically Modulates the Hormone-Dependent Transcriptional Activity of ER and PR

A, HeLa cells were transiently transfected with receptor expression plasmid for PR, ER, and their cognate hormone response elements in the presence or absence of WBP-2. Later, cells were treated with respective hormones as follows: PR, progesterone (10^{-7} M); ER, estradiol (10^{-8} M). Cells were harvested after 24 h and assayed for luciferase activity, and *bars* represent mean from three different determinations. The data are presented as fold activation. The activity of each receptor in the presence of hormone and in the absence of WBP-2 was defined as 1-fold, and the data for other *bars* were scaled accordingly. B, WBP-2 has no significant effect on the hormone-dependent transcriptional activity of AR and GR. LNCaP cells were transfected with androgen-responsive reporter in the presence or absence of WBP-2. HeLa cells were transiently transfected with GR expression plasmid and its cognate hormone response elements in the presence or absence of WBP-2. Later, LNCaP and HeLa cells were treated with R1881 (2.5×10^{-10} M) for AR and dexamethasone (10^{-7} M) for GR, respectively. Cells were harvested after 24 h and assayed for luciferase activity, and *bars* represent mean from three different determinations. The data are presented as fold activation. The activity of each receptor in the presence of hormone and in the absence of WBP-2 was defined as 1-fold, and the data for other *bars* were scaled accordingly. C, WBP-2 enhances PR transactivation in T47D cells. T47D cells were transiently transfected with progesterone response element containing reporter plasmid in the presence or absence of WBP-2. Later, cells were treated with progesterone (10^{-7} M). Cells were harvested after 24 h and assayed for luciferase activity, and *bars* are mean and SD from three different determinations. The data are presented as fold activation. The activity of receptor in the presence of hormone and in the absence of WBP-2 was defined as 1-fold, and the data for other *bars* were scaled accordingly. D, WBP-2 enhances ER transactivation in MCF-7 cells. MCF-7 cells were transiently transfected with estrogen response element containing reporter plasmid in the presence or absence of WBP-2. Later, cells were treated with either vehicle (−H), estradiol (10^{-8} M), or antiestrogen, tamoxifen (10^{-6} M). Cells were harvested after 24 h and assayed for luciferase activity, and *bars* are mean and SD from three different determinations. The data are presented as fold activation. The activity of receptor in the presence of hormone and in the absence of WBP-2 was defined as 1-fold, and the data for other *bars* were scaled accordingly. E, WBP-2 had no significant effect on the transcriptional activity of nonnuclear hormone receptor transcription factors p53 and VP-16 activation domain. HeLa cells were transiently transfected with expression plasmid for either p53 or VP-16 activation domain along with their respective reporter plasmids, p21 promoter-LUC or 17-mer-LUC in the presence or absence of WBP-2. Data are expressed as mean and SD of three different transfection assays. The data are presented as fold activation. The activity of each transcription factor in the absence of WBP-2 was taken as 1-fold, and the other *bar* was scaled accordingly.

with PR and PR-responsive reporter plasmids. Expression of siRNA directed against WBP-2 reduced the level of WBP-2 expression, whereas the control siRNA had no effect on WBP-2 expression (Fig. 4A). Depletion of endogenous WBP-2 reduced PR transcriptional activity by 75.3% (Fig. 4B), indicating that WBP-2 is required for fully functional PR activity.

We further investigated whether disruption of WBP-2 expression also affects estrogen-dependent biological actions in ER-positive MCF-7 breast cancer cells. MCF-7 cells were transfected with two control siRNAs (siScrambled or siGAPDH) or siWBP-2, and then cells were treated with either vehicle or estradiol (E2) and expression of estrogen-regulated gene, pS2 was measured by quantitative RT-PCR. Upon treatment with E2, pS2 mRNA was significantly induced in cells that were transfected with either of the control siRNAs (siScrambled or siGAPDH) (Fig. 4C). Importantly, cells treated with siWBP-2 had a reduced *pS2* expression, indicating WBP-2 is required for biological activity of ER in MCF-7 cells (Fig. 4C).

Recruitment of WBP-2 onto an Estrogen-Responsive Promoter in MCF-7 Cells

To further substantiate the coactivation function of WBP-2, we employed chromatin immunoprecipitation (ChIP) to assay the recruitment of WBP-2 onto an ER-responsive promoter in MCF-7 cells. Formaldehyde cross-linked protein-chromatin complexes were immunoprecipitated from MCF-7 that were treated with or without estrogen using the appropriate specific antibodies. The precipitated genomic DNA associated with ER or WBP-2 was amplified by PCR using primers specific for estrogen receptor binding site within the pS2 promoter. ChIP analyses demonstrated the recruitment of WBP-2 onto ER-responsive promoter in the presence of estrogen (Fig. 5, A and B); thus, as is the case for known ER coactivators, WBP-2 is recruited to the pS2 promoter in a hormone-dependent manner (29). This assay also demonstrates that WBP-2 is physically present on an ER target gene promoter at a time when the gene is activated.

WBP-2 Reverses Transcriptional Interference between Estrogen and Progesterone Receptors

Previously, it has been demonstrated that the transcriptional activity of one receptor can be squelched by the overexpression of another receptor, indicating that both receptors compete for limited pools of common factors (21, 30). We used a transient transfection assay to determine whether WBP-2 can relieve ER-induced squelching of the transcriptional activity of PR. We observed that PR-mediated transcriptional activity was reduced by 75% upon coexpression of ER in the presence of estradiol (Fig. 6, compare lanes 2 and 3). Addition of WBP-2 reversed this squelching in a dose-dependent manner (Fig. 6, compare lanes 2 and 8). In control cells that are not treated with estradiol,

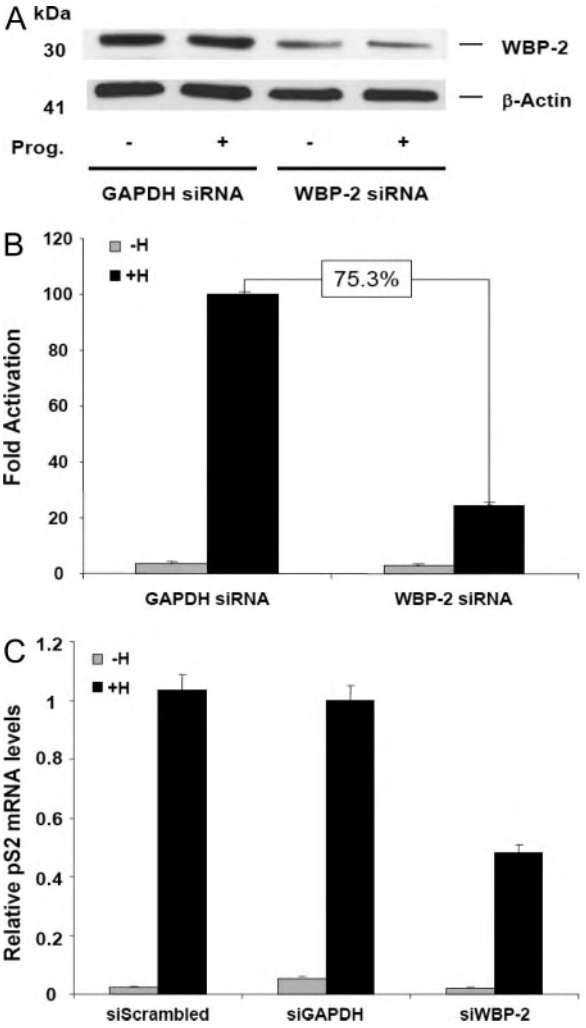

Fig. 4. Endogenous WBP-2 Expression Is Essential for Steroid Hormone Receptor Function

A, Expression analysis of WBP-2 protein after siRNA treatment. HeLa cells were transiently transfected with PR expression plasmid and its response element either in presence of WBP-2 siRNA (*in vitro* synthesized) or control GAPDH siRNA. Four hours post transfection, cells were treated either with progesterone (10^{-7} M) or vehicle (ethanol). Twenty-four hours after transfection, cells were harvested and lysed. Cell lysates were run on a 4–20% gradient gel and transferred onto nitrocellulose paper. Protein levels were assessed by Western blot using WBP-2-specific antibodies. Equal loading of samples was confirmed using β-actin-specific antibodies. B, Depletion of endogenous WBP-2 levels reduces transcriptional activity of PR. Part of the cell lysates was used to measure luciferase activity. The activity of PR in the presence of progesterone and control siRNA (GAPDH) was taken as 100-fold activation, and the data for other *bars* were scaled accordingly. C, Depletion of endogenous WBP-2 levels reduces the expression levels of ER target gene pS2. MCF-7 cells were transfected with either control siRNA (siScrambled), siGAPDH, or siWBP-2. Then, cells were treated with vehicle (−H) or E2 (+H) for 12 h before being collected for RNA preparation. RT-PCR was performed with specific primers for *pS2*. The results were normalized against GAPDH transcripts, and plotted as relative fold mRNA levels of pS2. The relative mRNA level of pS2 gene when treated with siRNA against GAPDH is taken as 1-fold, and other *bars* are scaled accordingly.

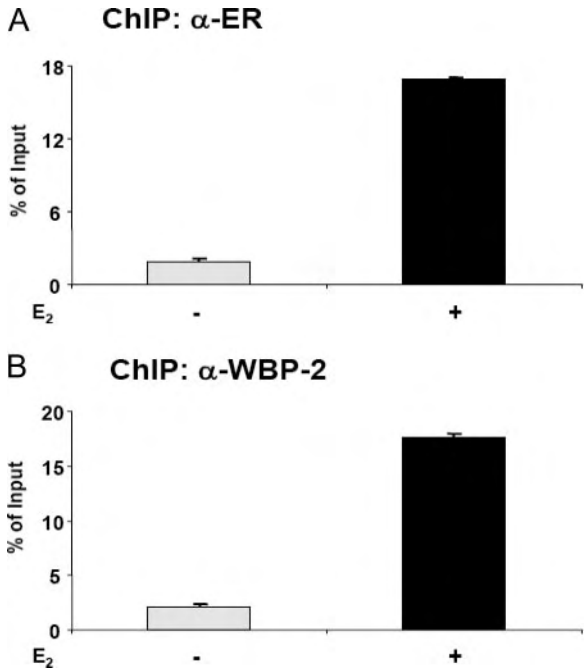

Fig. 5. WBP-2 Is Recruited onto ER-Responsive Promoter

ChIP was performed using MCF-7 cells in the presence of E2 (+) or absence of estrogen hormone (−). A, Primers specific for pS2 promoter were used to amplify the genomic DNA associated with ER in MCF-7 cells. B, Primers specific for pS2 promoter were used to amplify the genomic DNA associated with WBP-2 in MCF-7 cells. The PCR products were separated on agarose gel and the band intensities were quantified using the Image J (NIH) software. Input band intensity was considered as 100%, and the other bands were scaled accordingly.

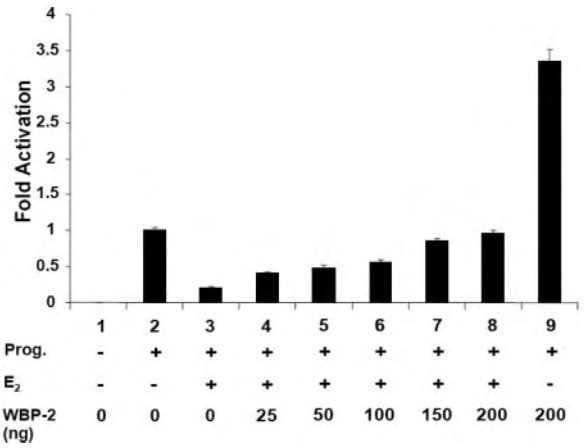

Fig. 6. WBP-2 Reverses the Transcriptional Interference between PR and ER

HeLa cells were transfected with 0.2 μg of PR expression plasmid, 0.3 μg of ER expression plasmid, 1.0 μg of PRE-.TATA.LUC, and increasing concentrations (0, 25, 50, 100, 150, 200 ng) of WBP-2. Cells were then treated with progesterone (Prog.) alone or progesterone and estradiol together (each at 10^{-8} M). The *last bar* corresponds to control cells transfected with WBP-2, ER and PR expression plasmids but treated with progesterone alone. Data are expressed as mean and SD of three independent transfections. The data are presented as fold activation. The activity in the presence of hormone and in the absence of WBP-2 was defined as 1-fold, and the data for other *bars* were scaled accordingly.

WBP-2 transfection increased PR-mediated transcriptional activity 3- to 4-fold (Fig. 6, compare lanes 2 and 9). Thus, coexpression of WBP-2 can reverse the interference between ER and PR, suggesting that WBP-2 is one of the limiting factors that are necessary for efficient PR and ER transcriptional activity.

The Carboxyl-Terminal PY Motif of WBP-2 Is Also Required for Its Coactivation Function

WBP-2 contains three proline-rich motifs containing the sequence PPXY (P, proline; X, any amino acid; Y, tyrosine) (21). These PPXY sequences or PY motifs in WBP-2 are binding recognition sites for a subclass of WW domains present in certain other proteins. Because WBP-2 enhances the transcriptional activities of ER and PR, we wanted to determine whether the PY motifs of WBP-2 are essential for its ability to modulate the transcriptional activity of these receptors. To this end, we mutated each of the three PPXY motifs of WBP-2 to AAXA (A, alanine) (Fig. 7A) and tested the ability of the mutant WBP-2 proteins to modulate PR transcriptional activity by transient transfection assays. As shown in Fig. 7B, wild-type WBP-2 enhanced the transcriptional activity of PR in a hormone-dependent manner in transient transfection assays. Similarly, the WBP-2 proteins that contained mutations in either PY motif 1 or PY motif 2 were able to enhance PR activity, suggesting that PY motifs 1 and 2 of WBP-2 were nonessential for its coactivation functions (data not shown). In contrast, a WBP-2 protein that contained mutations in the carboxyl-terminal PY motif of WBP-2 was unable to coactivate the transcriptional activity of PR, suggesting that this PY motif of WBP-2 is required for its transcriptional function (Fig. 7B). To confirm that the loss of coactivation function in the carboxyl-terminal PY motif mutant WBP-2 was not due to the loss of expression of the mutant WBP-2, we analyzed the expression of mutant WBP-2 by Western blot analysis. Figure 7C demonstrates that both the wild-type and mutant WBP-2 proteins are expressed at approximately equal levels. To further ascertain that this loss of coactivation function of the carboxyl-terminal PY motif mutant WBP-2 is not due to its lack of interaction with the receptor, we also analyzed the interaction between wild-type WBP-2 and PY3 motif mutant WBP-2 with ER in GST pull-down assays. As shown in Fig. 7D, both wild-type WBP-2 and PY motif mutant WBP-2 interact with ER in a hormone-dependent manner, suggesting that loss of coactivation function of mutant WBP-2 is not due to loss of its interaction with receptor. Taken together, these results demonstrate that the carboxyl-terminal PY motif of WBP-2 protein is critical for its ability to enhance steroid hormone receptor-mediated transcription.

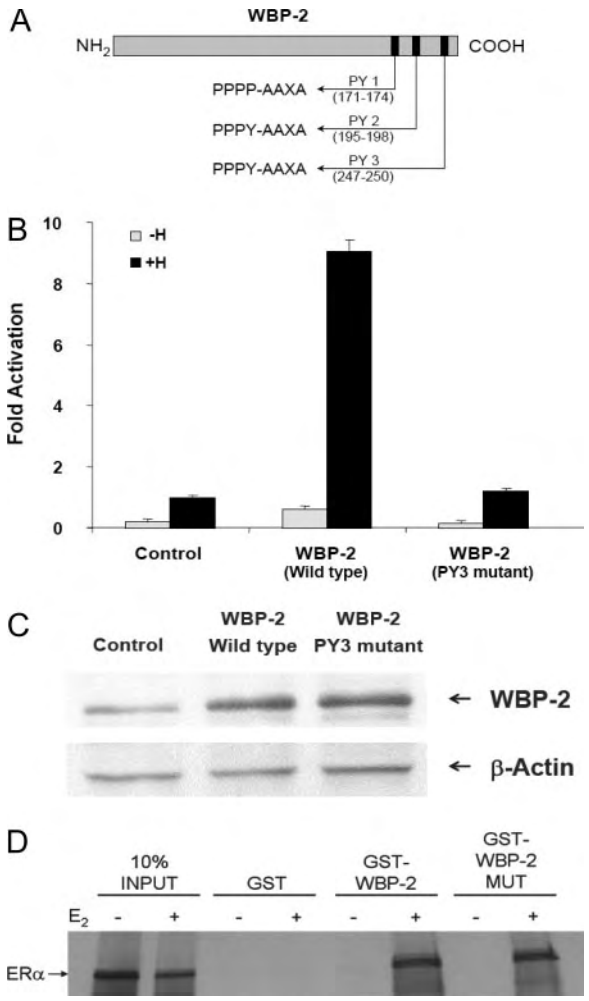

Fig. 7. PY Motif 3 of WBP-2 Is Indispensable for Its Coactivation Function

A, Schematic representation of mutated WBP-2 PY motifs. The PY motif 1, 2, and 3 of WBP-2 were mutated by *in vitro* site-directed mutagenesis. B, The PY motif 3 of WBP-2 is required for its coactivation function. HeLa cells were transfected with PR expression and reporter plasmids in the presence or absence of either wild-type or PY3 mutant WBP-2. Cells were treated with progesterone (10^{-7} M), and luciferase activity was measured. The data are presented as fold activation. The activity of PR in the presence of hormone and in the absence of WBP-2 was defined as 1-fold, and the data for other *bars* were scaled accordingly. C, Wild-type and PY3 mutant WBP-2 expressed at an equal level. Protein levels were analyzed by Western blot using anti-WBP-2 antibody. Control lane represents cells that were transfected with empty vector. β-Actin expression was used as loading control. D, Wild-type and PY3 mutant WBP-2 interact with ER. Interaction of wild-type and PY3 mutant WBP-2 with ER was determined in a GST pull-down assay. ER was labeled with ^{35}S by *in vitro* transcription and translation kit. The labeled ER protein was then incubated overnight at 4 C with *E. coli* expressed GST alone (control), GST-WBP-2 (wild type), or GST-PY3 mutant WBP-2 bound to beads either in the absence of hormone (−H) or presence of E2. Bound proteins were analyzed by SDS-PAGE and autoradiography with 10% of *in vitro*-translated ER as input.

The Carboxyl-Terminal PY Motif of WBP-2 Is an Integral Part of Its Intrinsic Activation Function

Authentic steroid hormone receptor coactivators often contain intrinsic transcription activation domains. To ascertain whether WBP-2 possesses an intrinsic transferable activation domain, wild-type WBP-2 and mutant WBP-2 (coactivation deficient) were fused to GAL4 DNA-binding domain. The ability of these fusion proteins to function as transcription activation domains was assayed using a GAL4-responsive reporter. In these experiments, we used GAL4-SRC-1, a well-characterized steroid hormone receptor coactivator as positive control. GAL4-WBP-2 (wild type) was able to stimulate the transcriptional activity of the reporter gene to a greater extent than did the vector containing only the GAL4 DNA-binding domain, whereas the activity of the coactivation deficient mutant WBP-2 was significantly reduced compared with that of wild-type WBP-2 (Fig. 8).

Coexpression of WBP-2 and E6-AP Further Enhances PR Activity

Because E6-AP has been reported to interact with steroid hormone receptors (16) and WBP-2, we wanted to further explore the functional interaction between E6-AP and WBP-2. HeLa cells were transiently transfected with wild-type E6-AP and WBP-2 expression plasmids either alone or together. WBP-2 and E6-AP each transfected alone significantly enhanced the activity of PR (Fig. 9). However, when coexpressed, WBP-2 and wild-type E6-AP additively enhanced the transactivation function of PR (Fig. 9). These data suggest that E6-AP and WBP-2 functionally cooperate with each other.

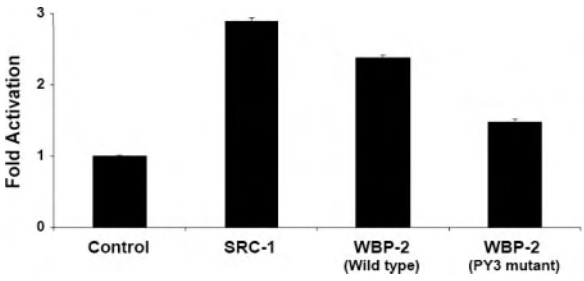

Fig. 8. PY Motif 3 of WBP-2 Is Essential for Its Intrinsic Activation Function

HeLa cells were transiently transfected with either pBIND (empty), pBIND-WBP-2 (wild type), or pBIND-WBP-2 (PY 3 mutant) and its specific reporter pGS5. pBIND.SRC-1 was used as a positive control. Cells were harvested after 24 h and assayed for luciferase activity, and *bars* are mean and SD from three different determinations. The data are presented as fold activation. The activity of Gal4 DNA binding domain (control) in the absence of WBP-2 (wild type and mutant) or SRC-1 was defined as 1-fold, and the data for other *bars* were scaled accordingly.

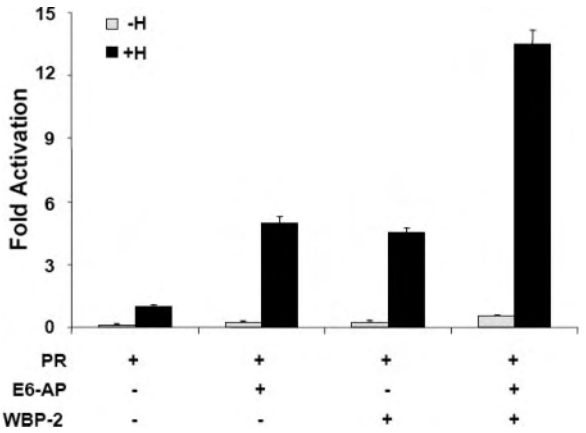

Fig. 9. WBP-2 and E6-AP Additively Enhance PR Transactivation

Cells were transiently transfected with PR expression plasmid and progesterone-responsive reporter plasmid (PRE.TATA.LUC) in the absence or presence of WBP-2 or E6-AP or both. Cells were treated with or without progesterone (10^{-7} M). Data are expressed as mean and SD of three different transfections and plotted as fold activation.

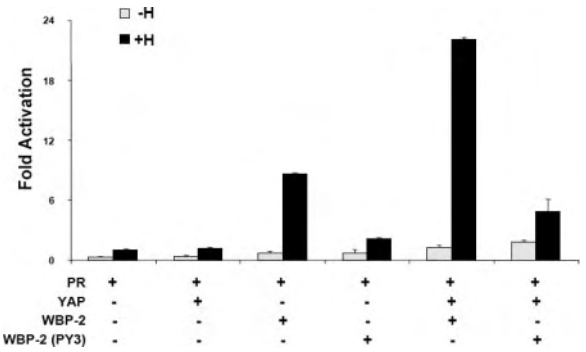

Fig. 10. YAP Shows an Absolute Dependence on Wild-Type WBP-2 to Enhance PR Transactivation

HeLa cells were transiently transfected with receptor expression plasmid for PR and its specific reporter (PRE.TATA.LUC). Wild-type WBP-2, mutant WBP-2 (PY3), and YAP expression vectors were also coexpressed alone or in combination. Empty vector of the above proteins was transfected as control. Later, cells were treated with progesterone (10^{-7} M). Cells were harvested after 24 h and assayed for luciferase activity, and *bars* are mean and SD from three different determinations. The data are presented as fold activation. The activity of receptor in the presence of hormone and in the absence of WBP-2/ mutant WBP-2/ YAP was defined as 1-fold, and the data for other *bars* were scaled accordingly.

The WW Domain-Containing Protein, YAP, Modulates Progesterone Receptor Transcriptional Activity via the WBP-2 Protein

WBP-2 was first identified as a protein-binding partner of the WW domain-containing protein, YAP. YAP has been shown to be a transcriptional coactivator (23). Thus, we wanted to know whether YAP may modulate steroid receptor-dependent target gene expression. To determine the role of YAP in steroid hormone receptor transactivation, HeLa cells were cotransfected with mammalian expression plasmids for the PR and ER receptors along with reporter plasmids containing their cognate hormone response element, with or without an expression vector for YAP. YAP did not affect PR-mediated transactivation either in the absence or presence of hormone. In contrast, when YAP was coexpressed with WBP-2, the hormone-dependent transcriptional activity of PR was significantly enhanced (~24-fold) (Fig. 10). Similarly, YAP alone did not activate the ER-mediated transactivation but when coexpressed with WBP-2 enhanced ER mediated transactivation (data not shown). This activity was higher than the observed coactivation with WBP-2 alone (Fig. 10). These data suggest that YAP can modulate the ligand-dependent transcriptional activity of PR and ER via WBP-2.

The PY motifs of WBP-2 have been shown to interact with the WW domain of YAP. Because our data revealed that the most carboxyl-terminal polyproline motif (PY motif 3) of WBP-2 was required for its coactivation function, we next asked whether the PY motif 3 of WBP-2 is required for YAP to function as a steroid receptor coactivator. When coexpressed together, wild-type WBP-2 and wild-type YAP greatly enhanced the transactivation function of PR (Fig. 10). In contrast, the mutant WBP-2 and wild-type YAP also failed to enhance the transcriptional activity of the progesterone receptor (Fig. 10). Our data demonstrate that the carboxyl-terminal PY motif of the WBP-2 protein also is required for YAP to function as a transcriptional secondary coactivator.

Discussion

In this study, we describe the isolation of WBP-2 as an E6-AP interacting protein and its role in transactivation by steroid hormone receptors. Our data demonstrate that WBP-2 interacts with E6-AP as well as with the liganded form of ER and PR both *in vitro* and *in vivo*. This result is consistent with previously published reports indicating that coactivators form multiprotein complexes (15, 29) and interact with receptors primarily in the presence of hormone. It has been suggested that most coactivators interact with receptors via the LXXLL motifs contained within the coactivators (31). WBP-2 is distinct from these coactivators, because it does not contain LXXLL motifs. It also has been reported that most of the cloned coactivators exhibit little receptor specificity and are able to coactivate a wide variety of nuclear hormone receptors. Unlike these coactivators, WBP-2 exhibits receptor selectivity and preferentially coactivates the hormone-dependent transcriptional activities of PR and ER, having little effect on the transactivation functions of GR and AR. In addition, as described for several recently char-

acterized coactivators (16), WBP-2 contains an intrinsic transactivation function.

Our ChIP analyses demonstrate the hormone-mediated recruitment of WBP-2 onto an endogenous ER-responsive pS2 promoter. Thus, as is the case for coactivator proteins such as E6-AP, UbcH7, and SRC family members, WBP-2 is also recruited to target promoters by receptors in a hormone-dependent manner. Because WBP-2 binds to both receptor and E6-AP, it is likely that WBP-2 could be recruited to the target promoters in a hormone-dependent manner by its association with receptors and/or E6-AP.

Targeted depletion of coactivators in mice and cell lines has demonstrated that coactivators are required for proper functioning of steroid hormone receptors (32, 33). Our siRNA-mediated depletion experiments suggest that, like other coactivators, WBP-2 is important for the proper functioning of steroid hormone receptors. The existence of modulatory proteins in the nuclear hormone receptor transactivation pathway is supported by the findings that the transcriptional activity of one receptor can be squelched by the overexpression of another receptor, indicating that both receptors compete for a limited pool of common factor. Our results indicate that overexpression of WBP-2 reverses the squelching effect of ER on PR transactivation in a dose-dependent manner and are consistent with previously published studies indicating that authentic coactivators usually can reverse squelching between two receptors (19). Taken together, our data support the observation that WBP-2 is a *bona fide* coactivator of PR and ER.

The WBP-2 protein contains three PPXY sequences known as the PY motifs. The PY motifs are present in the transcriptional activation domains of several transcription factors, including c-Jun, AP-2, C/EBPα, NF-E2, KROX-20, MEF2B, and PEBP2, suggesting that the PY motifs may play vital role in gene transcription. PY motifs in these proteins have been previously shown to mediate protein-protein interactions and they represent potential transactivation domains that could function by recruiting additional strong transactivators to the promoters of target genes. Our data support the hypothesis that the PY motif is involved in gene transcription because mutations in one of the three PY motifs abolish both the intrinsic activation function of the molecule as well as the coactivation function of WBP-2 in ER- and PR-mediated gene transcription.

Initially, WBP-2 was proposed as a ligand for the WW domain of YAP. In this report, we have shown that YAP also acts as a coactivator of steroid hormone receptors. However, the coactivation function of YAP is dependent on the presence of WBP-2 (carboxyl-terminal PY motif of WBP-2). Our data are consistent generally with previously published reports that YAP stimulates gene transcription by binding to the PY motif of ErbB4 protein. In conclusion, the results presented in this study substantiate the role of WBP-2 (contains PY motif) and YAP (contains WW domain) in female steroid hormone receptor function. Based on our data, we postulate that the PY motif of WBP-2 binds to the WW domain of YAP and recruits YAP to the target gene promoter by interacting with receptor and E6-AP. When the receptor-E6-AP-WBP-2-YAP complex is recruited to hormone-responsive promoters, it acts at one of the many substeps required to modulate the transactivation functions of a steroid hormone-responsive target gene.

Materials and Methods

Plasmid Construction

The mammalian expression plasmids for progesterone receptor-B (pCR3.1.PR-B), glucocorticoid receptor (pCR3.1.GR), estrogen receptor (pCR3.1.ER), p53, SRC-1 (pBIND.SRC-1), E6-AP (pCR3.1.E6-AP), YAP65 (pCDNA3.1.YAP65), pGEM.E6-AP (3003), and GAL-VP16 have been described previously (1, 16, 22, 34, 35). The progesterone/glucocorticoid/androgen-responsive reporter (PRE-TATA.LUC), estrogen-responsive reporter (ERE-TATA.LUC), p53-responsive reporter (p21 promoter-LUC), and VP-16-responsive reporter (17mer-LUC) plasmids also have been described previously (17, 36–38).

To reconstitute the ubiquitin-protein ligase defective E6-AP in a yeast two-hybrid plasmid, *Hin*dIII-digested (and filled) pGEM E6-AP (C833S) was redigested with *Bam*HI. The resulting *Bam*HI-*Hin*dIII (filled) fragment was inserted into the *Bam*HI-*Eco*RI (filled) sites of pGAD10 (Clontech, Cambridge, UK). To fuse WBP-2 with GST, the *Bam*HI-*Eco*R1 fragment of full-length WBP-2 was subcloned in-frame with GST into plasmid pGEX4T (Amersham Biosciences, Piscataway, NJ). To fuse WBP-2 in-frame with Gal4 DNA binding domain, the *Bam*HI-*Eco*RI fragment of WBP-2 was subcloned into the corresponding sites of pBIND (Invitrogen, San Diego, CA) vector and pBK-RSV (Stratagene, La Jolla, CA) vector. The reporter plasmid pGS5 with multiple copies of Gal4 response element was purchased from Invitrogen.

Site-Directed Mutagenesis of the PY Motifs of WBP-2

The GeneEditor *in vitro* Site-Directed Mutagenesis System from Promega Corporation (Madison, WI) was used to generate WBP-2 PY motif mutants. Mutations within the three poly-proline motifs (PY motifs) were generated in pBlueScript vector. The oligonucleotide primers used in the process are as follows: 5′-GGA ATG TAC CCC TGC GCT GCT GGC GCC CCC TAT CCA CCG CCC-3′ coding for AAGA (first PY motif); 5′-TAC GTG CAG CCC CCA GCA GCG CCC GCC CCT GGG CCC ATG GAA-3′ coding for AAPA (second PY motif); 5′-AGC CAG CCG CCG CCA GCT GCC TAC GCC CCA CCG GAA GAT AAG coding for AAYA (third PY motif). Mutants were screened and confirmed by sequencing. The cDNAs of PY mutants of WBP-2 were then digested with *Bam*HI-*Eco*RI and inserted into the corresponding sites of pBK-RSV, pBIND (in-frame with Gal4 DNA binding domain), and pGEX-4T (in-frame with GST).

Yeast Two-Hybrid Screening

The yeast two-hybrid screening assay was performed as described previously (16). A catalytically inactive form of E6-AP (C833S), in which the active site cysteine residue is substituted with serine, was used as bait. The prey cDNA library fused to the Gal4 activation domain was derived from human brain cells (Clontech).

WBP-2 Antibody Generation and Western Blot Analysis

Alpha Diagnostic International (San Antonio, TX) generated the antibody against WBP-2 protein. A unique 17 amino acid (N′-NDMKNVPEAFKGTKKGT-C′) peptide sequence was selected within WBP-2 protein based on hydrophilicity, antigenicity, and accessibility scale using various bioinformatics protein-profiling programs. Cross-reactivity of the peptide sequence was checked by basic local alignment search tool (BLAST) analysis and was conserved in WBP-2 proteins across species. This peptide was synthesized *in vitro* and covalently attached to a carrier protein (KLH) via a cysteine residue added to the amino terminus and was injected into rabbits for polyclonal antibody generation. Antibodies generated were concentrated by affinity purification. Anti-WBP-2 was used at 1:500 dilutions in immunoblotting, and at 1:50 for ChIP assay.

In Vitro Interaction Assay

In vitro expression of radiolabeled E6-AP and ER were performed by *in vitro* transcription and translation from rabbit reticulocyte extract in the presence of [^{35}S]methionine according to the manufacturer's recommended conditions (Promega). GST-WBP-2 was expressed in *Escherichia coli* DH-5α cells and purified on glutathione-Sepharose beads. The purified and glutathione-bound WBP-2 was incubated with *in vitro*-translated E6-AP and ER in NETN buffer [50 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), 0.1% Nonidet P-40] overnight at 4 C. After washing four times with NETN buffer, WBP-2-bound E6-AP and ER were eluted and separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Immunoprecipitation Assay

Twenty-four hours after growth, cells were washed in TEN buffer [40 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl] and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer containing salt [400 mM NaCl, 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonylfluoride (10 μl/ml), aprotinin (30 μl/ml), and 100 nM sodium orthovanadate (10 μl/ml)] by pipetting up and down. Thereafter, cell lysates were placed on ice for 30 min. To bring the salt concentration of cell lysates to 150 mM NaCl, 150 μl of NaCl-free RIPA buffer [1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonylfluoride (10 μl/ml), aprotinin (30 μl/ml), and 100 nM sodium orthovanadate (10 μl/ml)] was added to the lysates. After centrifugation at 4 C (21,000 × *g*), lysates were incubated with 20 μl of protein A-Sepharose and rocked at 4 C for 30 min. After centrifugation, supernatants were transferred to fresh tubes and lysates were mixed either with serum or anti-ER antibody (HC-20; Santa Cruz Biotechnology, Santa Cruz, CA) at 4 C for 2 h on a rocker. Afterward, 20 μl of protein A-Sepharose beads were added, and lysates were incubated for an additional hour at 4 C on a rocker. Finally, after extensive washing with NaCl-free RIPA buffer, immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blotting using either an anti-ER, anti-E6-AP, or anti-WBP-2 antibody.

Transient Transfection

HeLa and MCF-7 cells were maintained in DMEM containing 10% fetal bovine serum. T47D and LNCaP cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. A total of 3×10^5 cells was plated 24 h before transfection in six-well plates containing 5% dextran-coated charcoal-stripped serum. Cells were transfected with the indicated amount of DNA using FuGene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN). After 4 h, cells were treated with the indicated hormones and harvested 24 h later. Luciferase assays were performed using Promega's luciferase assay system.

Design, Construction, and Transfection of siRNA against WBP-2

The siRNA target finder program from Ambion (Austin, TX) was used to design siRNA against WBP-2. The sequences used are as follows from the amino terminus: Seq#1(AS) 5′-AACGTGCCAGAAGCCTTCAAACCTGTCTC-3′, Seq#1(S) 5′-AATTTGAAGGCTTCTTCTGGCACGCCTGTCTC-3′. Ambion siRNA construction kit was used to construct and purify scrambled and WBP-2-specific siRNA molecules for transfection assays. JetSI transfection reagent (Qbiogene/MP Biochemicals, Carlsbad, CA) was used for transfection in HeLa and MCF-7 cells followed by luciferase reporter assay (described previously) and real-time PCR analysis (described below), respectively. The GAPDH (control) siRNA was purchased from Ambion.

Real-Time PCR Analysis

The real-time PCR analysis was carried out according to previous studies in MCF-7 cells. RNA was isolated using the TRIzol (Invitrogen) reagent as per the supplier's protocol. cDNA synthesis (iScript cDNA Synthesis kit) and real-time PCR analysis (iQ SYBR Green Supermix) were performed using the protocol provided with the products (Bio-Rad, Hercules, CA). The DNA was quantified by real-time quantitative PCR using pS2 promoter-specific primers (forward, 5′-GCGCCCTGGTCCTGGTGTCCAT-3′; reverse, 5′-GAAACCACAATTCTGTCTTT CAC-3′). The results were normalized to PCR product amplified with a pair of GAPDH-specific primers (forward, 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse, 5′-GAAGATGGTGATGGGATTTC-3′). Real-time PCR were performed using the iCycler iQ multicolor real-time PCR detection system (Bio-Rad). To avoid variations from different samples, the relative *pS2* mRNA levels were normalized against GAPDH mRNA content of the same sample.

ChIP

The ChIP analysis was performed as described previously using MCF cells. The DNA was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and eluted in 50 μl of H_2O. Total input samples were eluted in 100 μl of H_2O and diluted 1:10 before PCR analysis. Each PCR contains 6 μl of immunoprecipitate or input, 0.5 μM of each primer, 0.4 mM dNTP mixture, 1× Titanium *Taq* PCR buffer (Clontech), and 1× Titanium *Taq*DNA polymerase (Clontech) in a total volume of 25 μl. The primers for the pS2 promoter were as follows: forward, 5′-GGCCATCTCTCACTATGAATCACTTCTGC-3′, and reverse, 5′-GGCAGGCTCTGTTTGCTTAAAGAGCG-3′. PCR was performed for 29 cycles with 1 min of denaturing at 94 C, annealing at 62 C, and extension at 68 C.

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